Invariant chain and internalization of mature MHC class II from the cell surface

Ole Jørgen Bjarnason Landsverk

Thesis for the degree
Master of Science

Department of Molecular Biosciences
University of Oslo
2005

Contents

Acknowledgements

The work presented in this study was carried out in Professor Oddmund Bakkes laboratory at the Department of Molecular Biosciences, University of Oslo, from March 2004 to June 2005.

I wish to thank my supervisor Professor Oddmund Bakke, for giving me the opportunity to do the work presented in this thesis, and for the guidance, encouragement and enthusiasm he has provided along the way. Thanks also to past and present members of Bakke lab, for all their help in solving the various problems I encountered with this project, and providing an inspiring environment to work in. Thanks to Ruby for introducing me to cell work, Frode for teaching me confocal microscopy, Trygve for help with the stable transfections, Cathrine and Shailly for lending me their expertise with cloning, Nicolas for many interesting discussions on immunology and Dmitrii for critical reading and helpful suggestions with the manuscript. A special thanks to my sister and father for sharing their experience and providing invaluable advice throughout my time in academia, and not least my mother for her persevering support and encouragement. Finally, I also wish to thank all my friends for persistently reminding me that there is more to life than science.

Oslo, August 2005

Ole J. B. Landsverk

List of abbreviations:

AAK1	Adaptor associated kinase-1
ADP	Adenosine diphosphate
AEP	Asparagine endopeptidase
AP	Adaptor protein
APC	Antigen presenting cell
BiP	Immunoglobulin binding protein
CALM	Clathrin assembly lymphoid myeloid leukemia
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
$CdCl_2$	Cadmium chloride
CIITA	Class II trans-activator
CLIP	Class II associated Invariant chain peptide
COPII	Coat protein complex II
CREB	Cyclic adenosine monophosphate (cAMP) response element (CRE) binding protein
DC	Dendritic cell
DEC	Dendritic cell expressed multilectin endocytic receptor (CD205)
EE	Early endosome
EEA1	Early endosomal antigen 1
EGFP	enhanced green fluorescent protein
Eps15	Epidermal growth factor receptor pathway substrate 15
ER	Endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
FYVE	PtdIns(3)P binding motif (the name is based on the first letters of four proteins containing this motif)
GGA	Golgi-localized, gamma-ear-containing, ADP-ribosylation-factor-binding proteins
GTPases	Guanosine triphosphatases
HA	Hemagglutinin
Hip	huntingtin-interacting proteins
HLA	Human leukocyte antigen
hMTIIA	Human metallothionein (MT) promoter
Hrs	Hepatocyte-growth-factor-regulated tyrosine-kinase substrate
Hsc70	Heat-shock protein family member
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
ILEV	Invariant chain induced large endosomal vesicles
imDC	Immature dendritic cell
LE	Late endosome
M1	Human fibroblast cell line
MAPK	Mitogen activated protein kinase
matDC	Mature dendritic cell
Mb	Megabase
MDCK	Madin Darby canine kidney
MelJuSo	Human melanoma cell line
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MRE	Metal response element
mRNA	Messenger ribonucleic acid
MT	Metallothionein
MTF-1	Metal response element-binding transcription factor-1
MVB	Multi-vesicular bodies
NFY	Nuclear factor-Y
NF-κB	Nuclear factor-κB
NK	Natural killer
NKT	natural killer T cell
P	Phosphate
PAGFP	Photo-activable green fluorescent protein
PAMP	Pathogen associated molecular pattern
PI-K	Phosphatidylinositol kinase
PKC	Protein kinase C
PM	Plasma-membrane
PRR	Pattern recognition receptor
PtdIns	Phosphatidylinositol
PTK	Protein tyrosine kinases
RFX	Regulatory factor X
RNA	ribonucleic acid
Snx	Sorting nexin
SV40	Simian virus 40
t-/v-SNAREs	Target-/vesicle-membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TcR	T cell receptor
TGN	Trans-golgi network
TH	T helper cell
TLR	Toll-like receptor
TM	Trans-membrane
wtGFP	Wild-type green fluorescent protein
X2BP	X2 binding protein

General Introduction

Complex multi-cellular organisms are subjected to a constant bombardment from a wide array of pathogenic agents. As a consequence they have evolved a large arsenal of defense mechanisms. The highly efficient first line of defense consists of the epithelial layers of the skin, and the mucosal surfaces of the gut and airways, which provide mechanical, chemical and also biological protection. Unfortunately, as evolution has provided us a means of defense, it has also provided the pathogens the ability to penetrate our defenses. The immune system has evolved to facilitate the removal of the pathogens that are successful in penetrating our outer parameters. It is generally divided into two parts, the innate and the adaptive immune systems, there is however considerable crosstalk between the two.

The innate immune system consists of an array of specialized cells which recognize general characteristics common to many pathogens (PAMPs; pathogen associated molecular patterns) through a wide array of cell surface receptors (PRRs; pattern recognition receptors). Cells of the innate immune system includes macrophages, neutrophils and natural killer (NK) cells, but also lymphocytes with an innate like behavior such as B1 cells (produce natural antibodies), epithelial γ:δ T cells (which recognize MHC-1B ligands) and natural killer T (NKT) cells (are activated in response to lipid antigens presented on CD1d). The complement also belongs to this system and consists of plasma proteins which act to opsonize pathogens and induce an inflammatory response.

The adaptive immune system comes into play at a later stage during the immune response. It is divided into two parts characterized by the production of antigen specific antibodies (the humoral) and the activation of antigen specific cytotoxic T cells (the T cell mediated). Both involve genetic recombination and selection, and both are highly specific for the pathogen encountered.

Adaptive immunity requires the presentation of pathogen derived peptides on the major histocompatibility (MHC) class I or class II molecules. MHC class I molecules are present on all nucleated cells and present peptides derived from pathogens resident in the cytosol. They react with the T cell receptor (TcR) on CD8+ T lymphocytes, alerting them

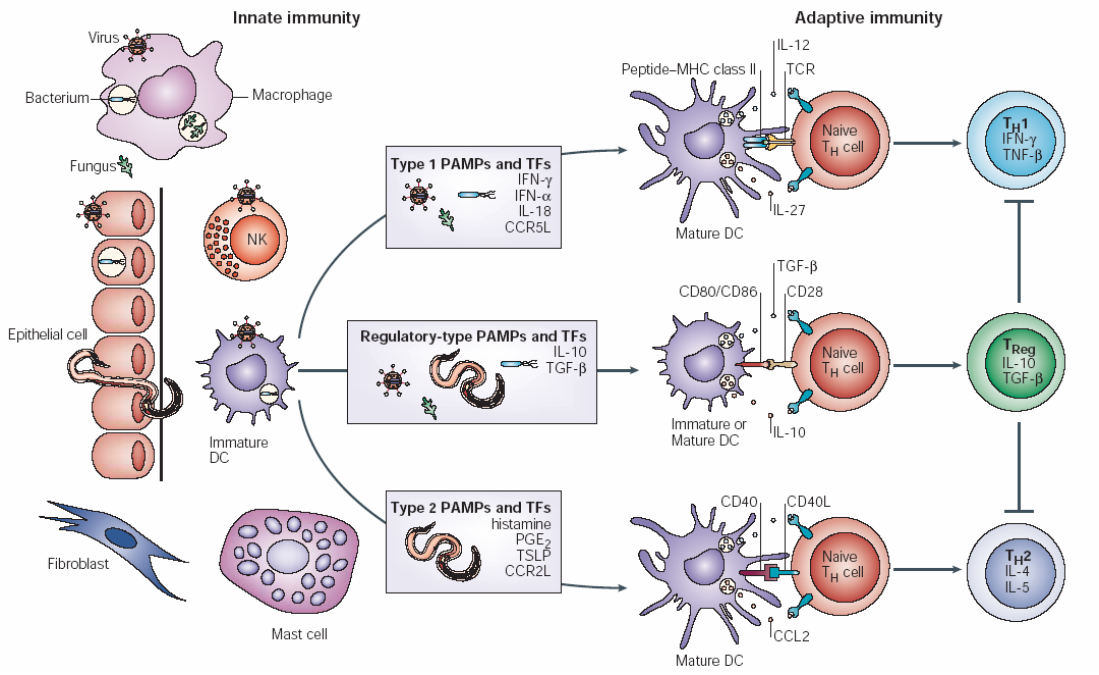

Figure 1. Differential activation of naïve T cells. Figure obtained from Kapsenberg, Nat Rev Immunol. (2003). Immature DCs polarized by type 1, type 2 and regulatory-type PAMPs or tissue factors (TFs) to become mature effector DCs that promote the development of naive T cells into TH1, TH2 or regulatory T cells. DC-polarizing tissue factors can be produced by various resident tissue cells and immune cells. These cells will produce type 1, type 2 or regulatory TFs depending on their origin or the way in which they are activated. TGF-β; transforming growth factor-β, CCL; CC-chemokine ligand, CCR; CC-chemokine receptor, PGE2; prostaglandin E2, TCR; T-cell receptor, TNF-β; tumour-necrosis factor-β.

to the presence of danger and provoking a cytotoxic response[1]. Expression of MHC class II molecules, on the other hand, is normally restricted to a limited number of cell types, termed "Professional Antigen Presenting Cells" (APCs)[2]. Their expression can however be induced on other cells in response to specific stimuli such as IFNγ. B cells are not considered to be very efficient APCs, the reason for which is that they only efficiently take up antigens recognized by their highly specific membrane bound IgM (mIgM). Considering the low frequency of specific B cell clones (before activation), the chance that they should encounter their respective T cell counterpart is extremely low. Macrophages however phagocytose pathogens indiscriminately and are therefore much more likely to present specific peptide MHC combinations recognized by many circulating T cells. Amongst APCs the Dendritic cells (DCs) are exceptional in their presentation capacity and warrants a special mention here. Many subpopulations of DCs have been characterized[3,4], with various varying functions attributed, but a simplified

description will suffice here. DCs reside or traverse throughout the various tissues of the body where they act as sentinels, constantly sampling their environment for danger signals. When activated by the encounter of antigen or cytokines, they undergo a remarkable differentiation, termed maturation. From being mostly tissue resident, they now display on their surfaces homing receptors for migrating to lymph nodes where large numbers of naïve T cells resides, and which they become the most potent activators of. They also display an astonishing morphological differentiation whereby their MHC class II molecules, which in immature DCs (imDCs) resides mostly intracellularly, is redistributed to the plasma membrane where they remain stably for several days. This imparts on these cells an unique ability to present an accurate "snapshot" of their environment at the time of activation, and reduces the potentially dangerous presentation of peptides present before activation[5].

MHC class II molecules present peptides derived from ingested pathogens processed in endocytic compartments to the TcR on CD4+ T lymphocytes. In combination with co-stimulatory molecules and polarizing factors they will drive the expansion of Ag specific T cell clones toward a TH1, TH2 or TH3/regulatory T cell response, depending on the environment at the time of activation and the consequent polarizing factors released (Figure 1). A TH1 response will promote a cytotoxic T cell response, whereas a TH2 response will drive the subsequent activation of specific B cells and consequently antibody production. A regulatory T cell response will repress an immune response and is essential in preventing autoimmunity[6,7]. The MHC class II molecules and especially the pathways involved in loading peptides onto these molecules is the theme of this thesis.

1.0 Introduction

MHC class II molecules present peptides derived from exogenous antigens to the TcR on CD4+ T lymphocytes. The compartments of the endocytic pathway presents ingested antigens to a progressively lower pH and greater concentrations of proteases[2]. The MHC class II molecule consists of an α and a β chain, which after translation and translocation into the endoplasmatic reticulum (ER), dimerizes, forming the heterodimeric class II molecule. In the ER MHC class II encounters the invariant chain (Ii), trimers of Ii associates with three class II heterodimers forming a nonameric complex. Ii plays an important role in the assembly and transport of the MHC class II molecules. Via its class II associated invariant chain peptide (CLIP) region it blocks the peptide binding groove of MHC class II, preventing premature peptide binding and stabilizing the class II molecule. Ii contains within its cytosolic tail two sorting signals which direct the complex to late endosomes and lysosomes[8]. In the absence of Ii, class II molecules mainly bind peptides in early endosomal compartments[9]. These are far less acidic than late endosomes/lysosomes and contain a more sparse array of, and lower concentrations of, proteases. It is therefore likely that they will present a much more limited array of peptides than will MHC class II transported by Ii. Compartments were peptide loading takes place are required to have several characteristics[10], rated highly amongst these is the presence of HLA-DM, which acts as a peptide editor, promoting the loading of tightly binding peptides[11]. In the following sections I will elaborate on the MHC class II molecules and the pathways employed by them. Special focus will be given to the Ii molecule and its unique capabilities in directing MHC class II transport.

1.1 The major histocompatibility complex

The MHC is a large gene complex encoding, amongst other molecules, the MHC class I and II glycoproteins and their accessory molecules (for a review see Horton *et al.* 2004[12]). It was first discovered in mice in 1936 for its potent effects on the immune response to transplanted tissues[13]. The first sequence based map of the human MHC, the human leukocyte antigen (HLA), was published in 1999[14] and consisted of a 3.6

megabase-pair (Mb) long sequence derived from many individuals with various HLA types, thereby producing a mosaic MHC haplotype. More recently, the boundaries of the HLA has been redefined, and the extended MHC (xMHC) now covers a total of 7.6 Mb on the short arm of chromosome 6[15]. In contrast to the TcR and immunoglobulin (Ig) locus, the MHC class II genes do not undergo the extensive genetic recombination essential for generating high affinity binding sites. In stead, the MHC contains a huge number of class I and II alleles, with varying binding specificities, each able to bind a wide array of peptides. In fact the human leukocyte antigen (HLA) B was recently confirmed to be the most polymorphic gene in the human genome[15], and over 100 HLA-DRβ alleles are known. Classical human MHC class II genes consist of HLA-DR, HLA-DP and HLA-DQ with high polymorphism within each. In the murine system two classical polymorphic MHC class II genes are described, I-A and I-E. The Ii gene does not reside within the MHC, it is in fact located on an entirely different chromosome (chromosome 5 in humans and 18 in mice).

1.2 Transcriptional control of MHC class II genes

Expression of MHC class II molecules and their accessory molecules is under highly stringent control by a variety of cis- and trans-acting elements (reviewed by Mach *et al.*[16]). The MHC class II proximal promoter region contains S, X1, X2 and Y boxes with sequences, orientations and spacing that are conserved for α, β, HLA-DM and Ii genes in various species ranging from zebra fish to mice and humans. There are however subtle variation within these cis-acting elements which may account for allelic differences in the expression levels of individual MHC class II genes[17,18]. In particular the Y box in the Ii gene is inverted with respect to the Y boxes of the other genes (HLA-DP/-DQ/-DR/-DM/-DO) and the spacing between the X and Y box and composition of the conserved S-X-Y module differs between HLA-DRA and Ii. This might affect the assembly of trans-acting factors and affect Ii promotor strength[19] resulting in the observed elevated expression of Ii as compared to MHC class II[20-23].

Several transacting factors acting on the MHC class II proximal promotor region have been identified, and three complexes are described. RFX is a trimeric nuclear complex

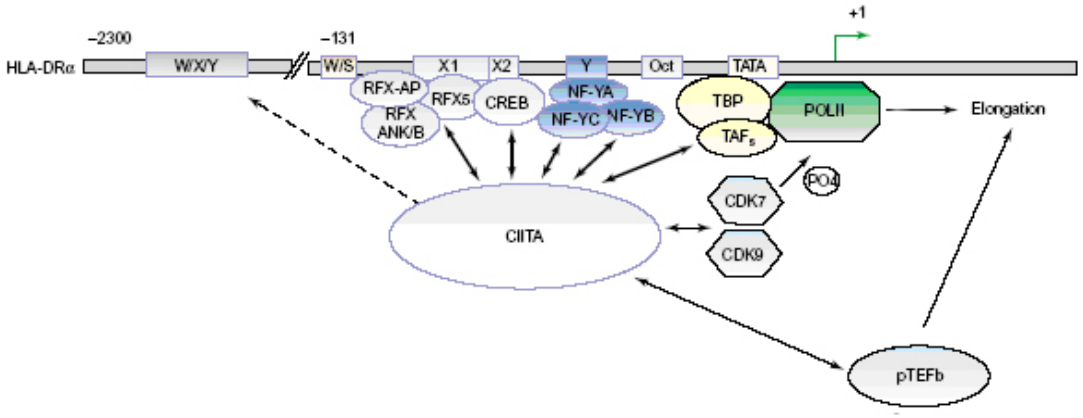

Figure 2 MHC-II enhanceosome on the HLA-DRa promoter. Obtained from Zika and Ting, Curr Opin Immunol. (2005). MHC-II promoters share a common set of cis-acting elements including the W/S, X1, X2 and Y boxes (S–X–Y module). The Y element binds the heterotrimeric transcription factor NF-Y. The X1 region is recognized by the RFX complex, whereas X2 is bound by CREB. These transcription factors are ubiquitously expressed and form an appropriate interaction surface for the recruitment of CIITA. CIITA also interacts with an array of TAFs and elongation factors such as pTEFb. Finally, CIITA interacts with the kinases CDK7 and CDK9, and enhances their ability to phosphorylate RNA polymerase II leading to initiation of mRNA synthesis.

that binds to the X-box[24], NF-Y is a heterodimer which binds CCAAT of the Y-box cooperatively with RFX[25] and X2BP, also a heterodimeric complex which includes CREB[26], binds the X2-box[27]. The three more or less ubiquitously expressed complexes bind cooperatively to the promoter forming a stable macromolecular nucleoprotein complex, termed the MHC II enhanceosome[28], onto which the class II trans-activator (CIITA) binds. CIITA is the master regulator of MHC class II genes[29,30] and controls the constitutive expression of MHC class II and accessory molecules[31-34] in APCs as well as the inducible expression in other cell types. If transfected into cells, CIITA can by itself drive the expression of the MHC class II genes. CIITA interacts with numerous cofactors to modify chromatin so that the ubiquitously expressed DNA binding complexes can assemble on the class II promoters and form the MHC II enhanceosome[35,36]. Once bound onto the enhanceosome, CIITA interacts with components of the basal transcription machinery to initiate transcription of target genes (Figure 2). Three different isoforms of CIITA resulting from the use of four different promoters (pI-pIV) have been described[37] with varying expression levels amongst APCs as well as IFNγ induced cells[29]. CIITA is naturally itself under stringent control at the transcriptional as well as epigenic level, and several factors have been described that activate, modulate or repress CIITA expression[29,38].

In addition to the transcriptional control of MHC class II expression as mediated by CIITA, there is evidence for further modulation of class II expression at the translational level. Stabilization of mRNA following activation with CpG has been described for HLA-DR in B lymphocytes[39] and there is evidence implying that also the Ii mRNA stability may be subject to regulation[40].

1.3 MHC class II molecules

MHC class II molecules are expressed as heterodimers of two transmembrane polypeptides (Figure 3). The α chain (~35kD) and a β chain (~27kD) differ in size mainly due to differences in N-linked glycosylation[41]. The α and β chain spontaneously associate in the ER due to interactions between their lumenal[42,43] and transmembrane[44] domains. All classical class II molecules have the same conformation, consisting of two extracellular domains α1 and α2, and β1 and β2 for the α and β chains, respectively. The peptide binding groove is composed of the membrane-distal α1 and β1 domains of the two chains, forming a platform of eight antiparallel β strands lined by two antiparallel α-helices[45] with a disulfide bond linking the α-helix of the β1 to the β sheet floor (β10C-β78C). The polymorphism of MHC class II molecules is largely located to these domains. The groove of class II molecules, as opposed to class I, is open at the ends, and the C- and N-termini of bound peptides may therefore extend beyond the ends of the groove. Conserved amino acid side chains lining the groove form hydrogen bonds to the peptide backbone[46], conferring sequence independent binding onto the class II molecule and enabling the high affinity binding of a wide range of peptides. There is, however, a requirement for the presence of certain residues in the

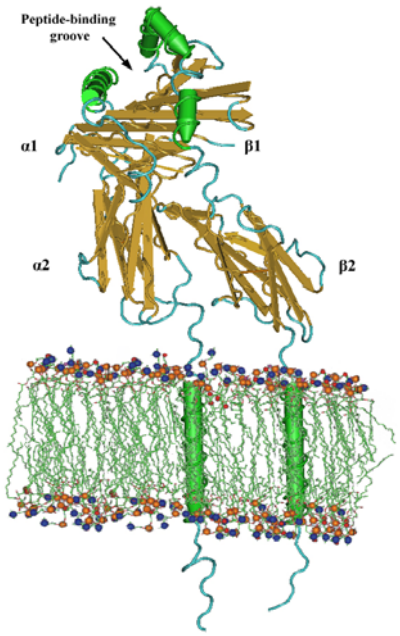

Figure 3. Model of MHC class II. The lumenal domain was taken from a crystal structure of HLA-DR1 (Zavala-Ruiz *et al*. 2003) the transmembrane and cytosolic domains are modelled. See text for details.

peptides presented by specific class II alleles because of polymorph anchor residues making specific interaction with the peptide, such as has been described lacking in HA binding to I-Ak[46]. The β chain of the human and mouse class II molecules contain leucine based sorting signals in their cytosolic tails[47,48] which are required for internalization and recycling of class II molecules at the plasma membrane[49]. Efficient assembly and transport of MHC class II is, however, greatly enhanced in the presence of Ii[50,51].

1.4 The Invariant chain molecule

Ii is a type II transmembrane glycoprotein that comes in several isoforms due to alternative translation initiation sites and alternative splicing (Figure 4). The dominant isoform in humans is p33, it has an N-terminal cytosolic tail of 30 amino acids, a transmembrane domain consisting of residues 31-56, and a C-terminal lumenal domain of 160 amino acids. The p35 form of Ii results from use of the alternative translation initiation site and adds 16 amino acids to the N-terminal cytosolic tail[52,53], these additional residues confer an ER retention signal (R-X-R) onto the isoform[54,55], which may be overcome through interaction with the cytosolic tail of the HLA-DRβ chain[56]. The p33 and p35 isoforms generate the p41 and p43 forms through alternative splicing of exon 6, resulting in a 64 residue insertion into the lumenal domain encoded by exon 6b[52,53,57]. Ii contains both N-linked and O-linked glycosylation sites and can be both

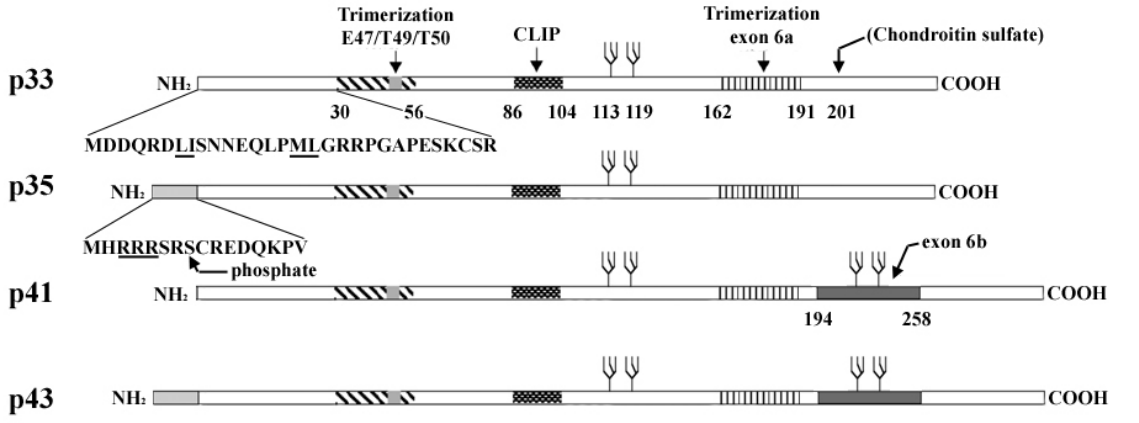

Figure 4. Invariant chain isoforms. Adapted from Gregers et al. Intracellular Pathogens in Membrane Interactions and Vacuole Biogenesis (2003). See text for details.

phosphorylated and modified by addition of chondroitin sulfate moieties (reviewed in[8,58,59]). Ii forms trimers in the ER due to trimerization domains located in the transmembrane (TM) segment[60] and in the lumenal domain C-terminal to CLIP (residues 163-183)[61,62](Figure 5). The biological significance of Ii trimer formation is not definitely established. Class II association and interaction with adaptor proteins proceeds normally if trimerization is inhibited[63], but presentation of certain peptides is abolished[64] indicating that trimerization is important for antigen presentation to T cells. Trimerization is however a requirement for formation of the Ii-induced enlarged endosomal vesicles[61] (ILEVs) and may therefore be involved in modulating the endocytic pathway to facilitate peptide loading. Ii is a multifunctional molecule and in addition to its conventional properties it has been suggested as a signaling factor through the cleavage of its cytoplasmic tail and activation of the NF-κB pathway (reviewed in[65]) as a chaperone for HLA-DM[66], and as a cell surface receptor for MIF[67]. This, however, lies beyond the scope of this text, and the reader is directed to the references for further details.

1.5 The MHC class II-Invariant chain complexes

Ii interacts with the class II heterodimer via several domains, the most prominent being the CLIP region of which the C-terminal part occupies the peptide binding groove, preventing premature peptide binding in the ER and golgi[68-73], and the N-terminal part binds outside the groove, contributing to the fast off-rate of CLIP[74,75]. Additional interacting regions must be present as it has been shown that Ii can interact with class II even when the peptide binding groove is occupied by high affinity peptides[76]. A region C terminal to CLIP (amino acids 103-118) has been shown to interact with class II under conditions where Ii has low affinity for the peptide binding site[77], two β-strands in the class II α chain form a binding pocket for Ii with a salt-bridge between the α chain residue 181 and Ii residue 74 and Ii amino acids 36-57 form electrostatic interactions with the class II β chain[78]. Also, the TM region of Ii has been suggested as an additional CLIP independent MHC class II interaction site[79]. The number of class II interacting domains that have been described so far, suggests that this interaction is of great evolutionary importance. After translocation into the ER membrane Ii and the class II α and β chains

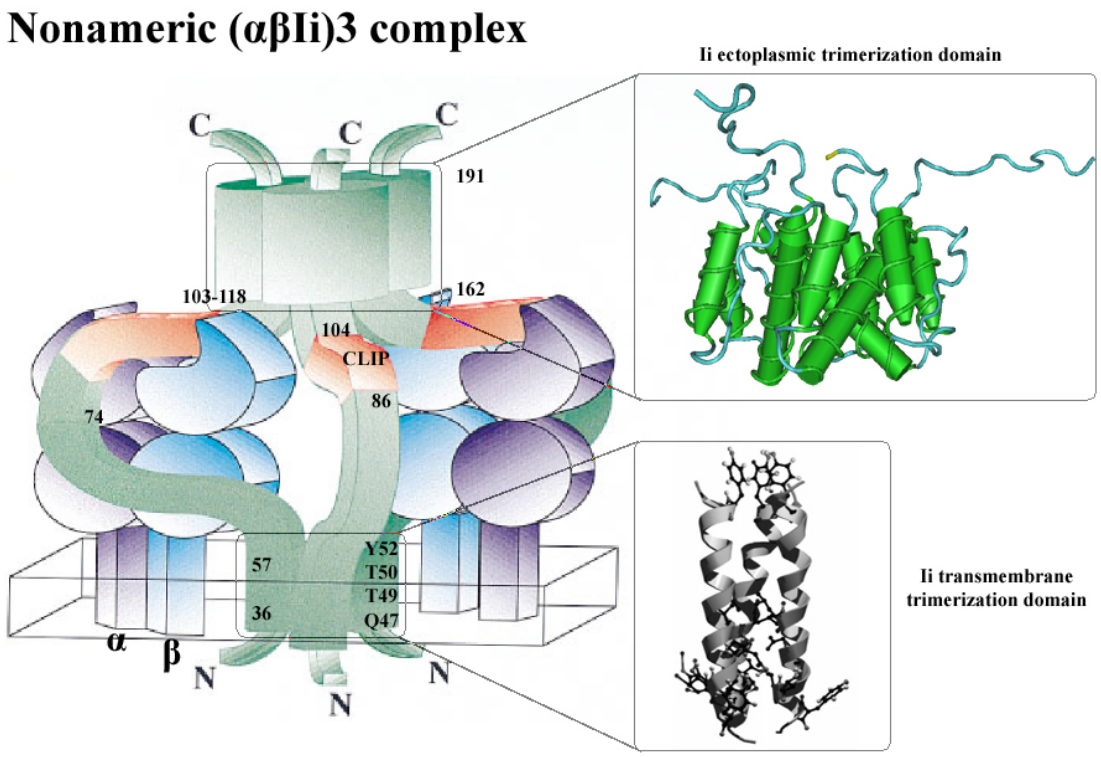

Figure 5. Nonameric (αβIi)3 complex. Adapted from Cresswell, cell. (1996), Kukol *et al.* Mol Biol. (2002) and Jasanoff *et al.* (1999). MHC class II interacting and trimerizing residues are indicated. The transmembrane trimerization motif is thought to be a cluster of polar residues (Q47, T49, T50, Y52) near the lumenal interface{Ashman, 1999 #228;Kukol, 2002 #743}. See text for further details.

associate into a nonameric $(\alpha\beta Ii)_3$ complex[80] (Figure 5). The order of assembly is not clear, and may differ for various class II alleles[81], Ii trimers either associate with pre-formed αβ dimers[80] or with α and β chains in a stepwise manner[82,83]. Ubiquitously expressed ER chaperones, such as Calnexin[84,85] and BiP[86], assist in the assembly of the $(\alpha\beta Ii)_3$ complex by associating with, and retaining, the free subunits. Correctly assembled complexes are freed from the ER quality control system and assemble in COPII coated vesicles for transfer to the golgi apparatus[87]. In the golgi, the $(\alpha\beta Ii)_3$ complexes undergo further modification of sugar side chains and are acetylated[88], sulfated[89] and phosphorylated[90]. These modifications are interdependent on the presence of Ii and class II, as αβ are poorly glycosylated in the absence of Ii[91], and Ii does not efficiently acquire complex sugars in the absence of class II[92]. The consequences of these modifications have not been conclusively established.

1.6 Other MHC class II accessory molecules

The non-classical MHC class II molecules HLA-DM and HLA-DO (H2-M and H2-O in mice) are both heterodimeric proteins consisting of an α and β chain. HLA-DO is highly similar to the classical class II molecules HLA-DR/-DP/-DQ (60% identical), whereas HLA-DM is further removed with as much similarity to class I as class II (approximately 30%)[11,93]. The majority of HLA-DM resides in multi-vesicular/-lamellar endosomes (collectively multi-vesicular bodies (MVBs) here for simplicity), but it has been detected throughout the endocytic pathway. The HLA-DM β-chain contains in its cytosolic tail a tyrosine based sorting motif (YPTL) which targets it to the endosomal system[94-96]. If this signal is mutated HLA-DM is blocked at the plasma membrane (PM), but can be rescued (transported to endosomes) if Ii is present[94]. HLA-DM lacks peptide binding properties, instead it interacts with MHC class II molecules, promoting the release of CLIP, stabilizing the empty heterodimers and facilitating the loading of high affinity peptides[97-100]. The interaction between HLA-DR and HLA-DM is of low affinity and sensitive to pH[101,102], and recent data suggest that it may be restricted to the internal structures of MVBs[103]. HLA-DO is, like HLA-DM, mainly localized to lysosomes[104-106] where it is associated with HLA-DM, mainly at the limiting membrane, but also on internal structures[103,107]. The long cytosolic tail of the HLA-DO β-chain contains two potential endosomal targeting motifs[108,109] which do not seem to function in intracellular transport[105], but may direct HLA-DO preferably to the limiting membrane of MVBs[110]. HLA-DO appears to be a pH dependent modulator of HLA-DM activity, at acidic pH HLA-DM/-DO complexes bind tightly to HLA-DR and enhance peptide loading[110], whilst at pH 6.0-6.5 loading is reduced[111,112], thereby promoting peptide loading preferably in late endosomes and lysosomes.

Another family of proteins shown to interact with class II molecules are the tetraspanins. Tetraspanins are four-pass transmembrane proteins containing 4 to 6 conserved extracellular cysteines linked via 2 or 3 disulfide bonds, and conserved polar residues within their transmembrane domains (reviewed in[113]). Tetraspanins such as CD63 and CD82 interact with HLA-DR and/or HLA-DM on the internal membranes of MVBs[114,115] and CD81 interacts with HLA-DR at the plasma membrane in B cells[116]. The functional

consequences of these interactions are not clear, but these proteins could be involved in the segregation and/or protection of class II and accessory molecules to facilitate peptide loading, and targeting and/or clustering MHC class II and co-stimulatory molecules to/in distinct membrane domains[117] to enhance antigen presentation.

2.0 Endocytosis and the endosomal system

As mentioned the endocytic pathway comprises a variety of compartments with a progressively wider array of and greater concentration of proteases. Entry into the endocytic pathway for exogenous components occurs via the plasma membrane and can be accomplished by a variety of means (Figure 6).

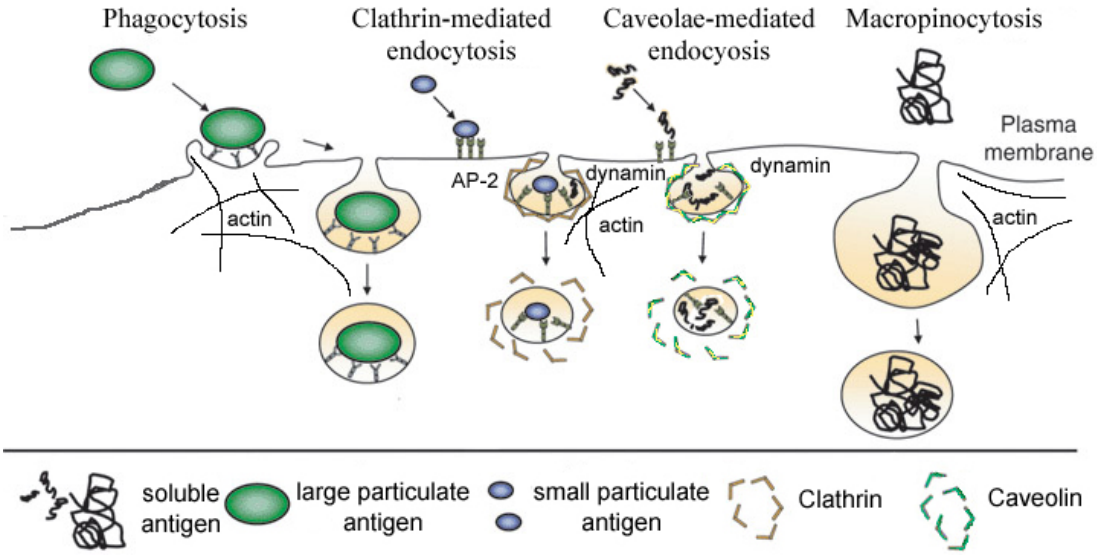

Figure 6. Types of endocytosis used for antigen acquisition. Phagocytosis involves the ingestion of large particles or cells. Particle binding to specific receptors signals actin assembly and drives pseudopod extension and particle engulfment. Macropinocytosis is also actin-dependent and can account for the uptake of large quantities of extracellular fluid and fluid-dissolved antigens. Soluble receptor-ligand complexes are typically internalized by clathrin-coated vesicles or caveolin-containing invaginations.

Phagocytosis is a complex process involving actin driven membrane protrusions engulfing particles and leading to the formation of large intracellular vacuoles. It requires receptor mediated recognition of the particle (or complement/antibodies attached to it) and can lead to several outcomes depending on the nature of the particle engulfed. Phagocytosis requires large amounts of membranes, and early endosomes are thought to

contribute to this, however, after internalization phagosomes may both obtain ER membranes and fuse with lysosomes. Toll-like receptors (TLRs), MHC class II and class I are all recruited to phagosomes, class II present peptides from phagocytosed antigens, and cross-presentation of exogenous antigens on class I is greatly enhanced if antigens are acquired by phagocytosis. The TLRs presumably play an important role (in collaboration with the initial recognizing receptors) in sampling of cargo and determining the consequent response (reviewed in references[118-120]).

Macropinocytosis occurs constitutively in immature DCs[121] allowing them to rapidly and non-specifically sample large amounts of extracellular fluid. Macropinocytosis, is like phagocytosis, an actin dependent process involving membrane ruffling, mediated by ras GTPases[122,123], and back-fusion of the membrane protrusion with the PM possibly involving rabs[124,125]. This generates macropinosomes which shrink and concentrate their soluble content. The maturation process occurs differently in different cell types, with both persevering homeotypic fusion of early endosomal antigen 1 (EEA1)-macropinosomes[126] and fusion with lysosomes described[127]. Antigens acquired by macropinocytosis can be presented on both MHC class I[128] and class II molecules[121].

Caveolae are small, cholesterol rich, hydrophobic membrane domains characterized by the presence of caveolin. They are present on all immune cells, and several pathogens are internalized via these flask-shaped invaginations[129]. Caveolae mediated endocytosis is triggered by signals resulting in the tyrosine-phosphorylation of caveolin and dynamin (a critical component of the fission machinery), and release of the caveolin coated vesicles to the cytosol[130-132]. Caveolar vesicles may fuse with caveosomes or dock on early endosomes (EE), if recruited by specific small GTPases[133], thereby rendering their content accessible to the antigen loading machinery.

Receptor mediated endocytosis via clathrin coated pits (CCPs) requires the ligation of antigen with specific receptors on the cell surface. The result is fast capture and effective concentration of a specific antigen[134] as specified by the receptors present on the particular APC. Targeting antigens to specific receptors such as DEC-205, which is preferentially expressed by DCs and internalized via CCPs, causes a 100- to 1000-fold more efficient antigen capture and presentation than soluble antigen[135,136]. The repertoire of receptors present is therefore of essential importance for antigen presentation.

Receptor ligation triggers the recruitment of a number of factors to the cytosolic face of the PM and as much of the same machinery is involved in the internalization of MHC class II and accessory molecules it will be elaborated on in the following section.

2.1 Cargo recruitment and membrane trafficking

Endocytosis via CCPs occurs via three mechanistically distinct intermediate stages characterized by a) assembly of the polygonal clathrin lattice defining the coated pits, b) invagination and c) pinching-off and liberation of clathrin coated vesicles (CCVs). Cargo-proteins are recruited into CCPs by binding via specific sequences in their cytoplasmic tails to adaptor proteins (APs), or other coat-associated proteins such as β-arrestin, but can also be marked for internalization by attachment of mono-ubiquitin[137]. AP-2 is, with clathrin, the most abundant protein found in CCPs, if AP-2 is knocked-down by RNAi the number of CCPs is reduced by over 90%[138,139], indicating its importance in CCP formation. AP-2 is a heterotetramer consisting of four subunits (α, β2, μ2 and σ2) which interact with numerous elements involved in CCP formation (Figure 7). The AP complexes appear to coordinate CCV formation by linking the mechanical clathrin coat to the phospholipid membrane, selecting and binding cargo and recruiting the plethora of other proteins involved in creating and disassembling a CCV's coat[140].

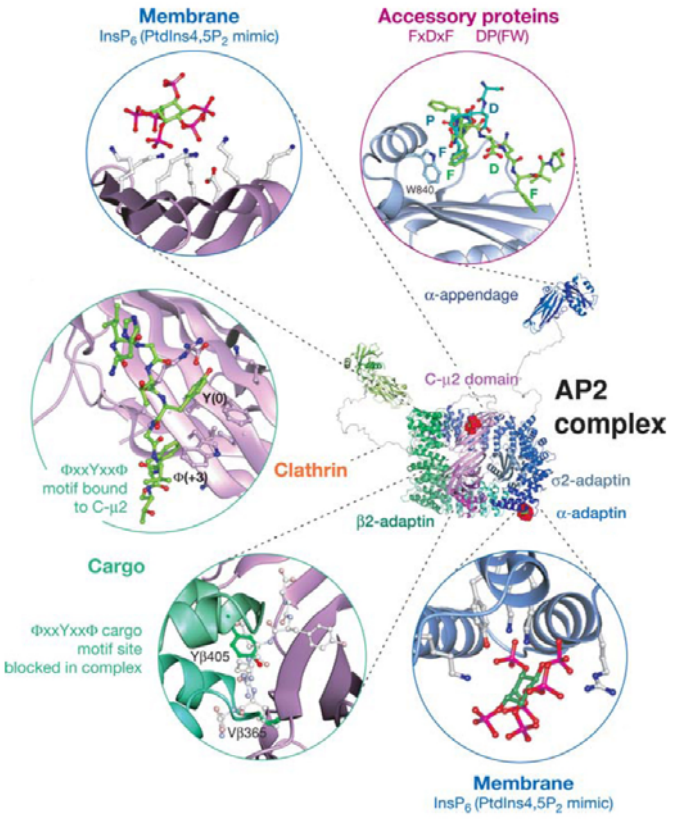

Figure 7. Model of AP-2 obtained from Owen *et al.* Annu Rev Cell Dev Biol. (2004). AP-2 subunits are colored *α* blue, *σ2* gray, *β2* green, and *μ2* purple. The appendages were solved separately, and the linkers connecting them to the core are modeled.

The α and μ2 domains contain PtdIns(4,5)P_2 binding sites for localization of AP-2 to the PM, whilst the μ2 domain recognizes YxxΦ motifs in the cytoplasmic tail of cargo proteins after AP-2 has undergone a conformational change brought on by phosphorylation by AAK1[141-143] and interaction with PtdIns(4,5) P_2. AP-2 is also thought to interact with acidic dileucine motifs ([DE]xxxL[IL]) in cargo proteins via its β2[144,145] and/or μ2[146] subunits and with a number of accessory proteins via their DP[FW], FxDxF or Wxx[FW] motifs to the α- and β2-appendanges of AP-2[140]. Clathrin interacts with a clathrin box motif (LΦxΦ[DE]) in the β2 subunit of AP-2[147]. The assembly unit of CCPs/CCVs is the clathrin triskelion, consisting of three heavy and three light chains arranged as a three-legged structure. Interaction with APs and other accessory proteins is mediated via a groove between

blades 1 and 2 of the 7-bladed β-propeller of the N-terminal domain of the heavy chains, however another non-competitive binding site exists on the top face of the β-propeller (W-box; binds a PWDLW motif present in the amphiphysins and Snx9)[148]. Clathrin assembles into lattices via intermolecular contacts between both the distal and proximal legs of the triskelions[149], there is however a requirement for generating membrane curvature in order to establish invaginations. This is accomplished through the recruitment of proteins and lipids[150] which act to deform the membrane. Amphiphysin, endophilin, dynamin and epsin are all able to deform membranes in vitro, and clathrin coated buds assemble onto them[151-153].

The precise order of assembly of the molecules forming CCPs and CCVs is not clear. A current modell of assembly, invagination and pinching of, suggest that epsin, CALM and Eps15 are involved in recruiting AP-2 to the PM. AP-2, though its various interacting domains, then recruit cargo, clathrin and other factors, and amphiphysin and endophilin directly promote invagination (and endophilin possibly fission) and target dynamin to CCPs. Dynamin is a GTPase which oligomerizes at the neck of invaginating pits, it is also involved in caveolae mediated endocytosis. Dynamin and also other CCP associated proteins (Intersectin, Hip1, Hip1R), may regulate actin polymerization and motor protein recruitment probably required for the final step of vesicle fission and propulsion of the nascent CCV into the cytosol, after which the clathrin coat is disassembled by Hsc70 and auxilin[154]. Freed vesicles attach via adaptor proteins to motor-proteins and traffic along

actin filaments and microtubuli to sorting or recycling EEs[155]. Fusion between CCVs and EEs involves specific rabs, phosphatidylinositol kinases (PI-Ks),docking factors and v- and t-SNAREs. Rabs act as molecular switches, cycling between inactive GDP-bound and active GTP-bound states. These changes in activity are coupled to the reversible association with their target membranes[156] and recruitment of PI-Ks and docking factors such as EEA1. The PI-Ks function to phosphorylate phosphatidylinositols creating docking sites for the various components of the fusion machinery[157-159]. EEA1 is a homodimer which binds via FYVE domains to PtdIns(3)P, and also to Rab5, on EE and functions as a docking factor for incoming vesicles[160], facilitating the interaction between t-SNARES (on target membranes) and v-SNARES (on docking membranes)[159], and the consequent fusion of vesicles with target compartments[161].

In EE cargo is selected for transport onwards to late endosomes and lysosomes or recycling back to the cell surface. The various other compartments within a cell are likewise interconnected through budding, fission, vesicular (and tubular) trafficking and fusion events, and the specific factors governing these events at specific locations differ, providing the necessary heterogeneity of the system[156,157,162].

Adding another layer of complexity to the endosomal system is the generation of intra-lumenal vesicles and, as MHC class II is intimately involved with these, it warrants a brief mention. Multi-vesicularity exists in varying degrees throughout the degradation pathway from EE, through late endosomes (LE) to lysosomes. Ubiquitinilation of the cytoplasmic domain of transmembrane proteins is the best described mechanism for sorting into MVBs. 'Hepatocyte-growth-factor-regulated tyrosine-kinase substrate' (Hrs) recruitment to membranes is dependent on PI3K activity[163], it recruits clathrin through a direct interaction[164] and binds to ubiquitin through its ubiquitin-interacting motif[165]. Hrs recruits 'endosomal sorting complex required for transport' (ESCRT)–I to endosomal membranes, and the sequential action of ESCRT-I, -II and –III is required for sorting into and formation of intralumenal vesicles[166]. The specific mechanism for sorting of MHC class II into intralumenal vesicles is not currently known.

2.2 MHC class II and Ii in the endocytic pathway

There has been some debate concerning the entry-point of the $(\alpha\beta Ii)_3$ complexes into the endocytic pathway from the trans-golgi network (TGN), and two main pathways have been described. The first postulates a direct transport from the TGN to a class II loading compartment (late endosome/lysosome related organelle)[167,168], whilst the second

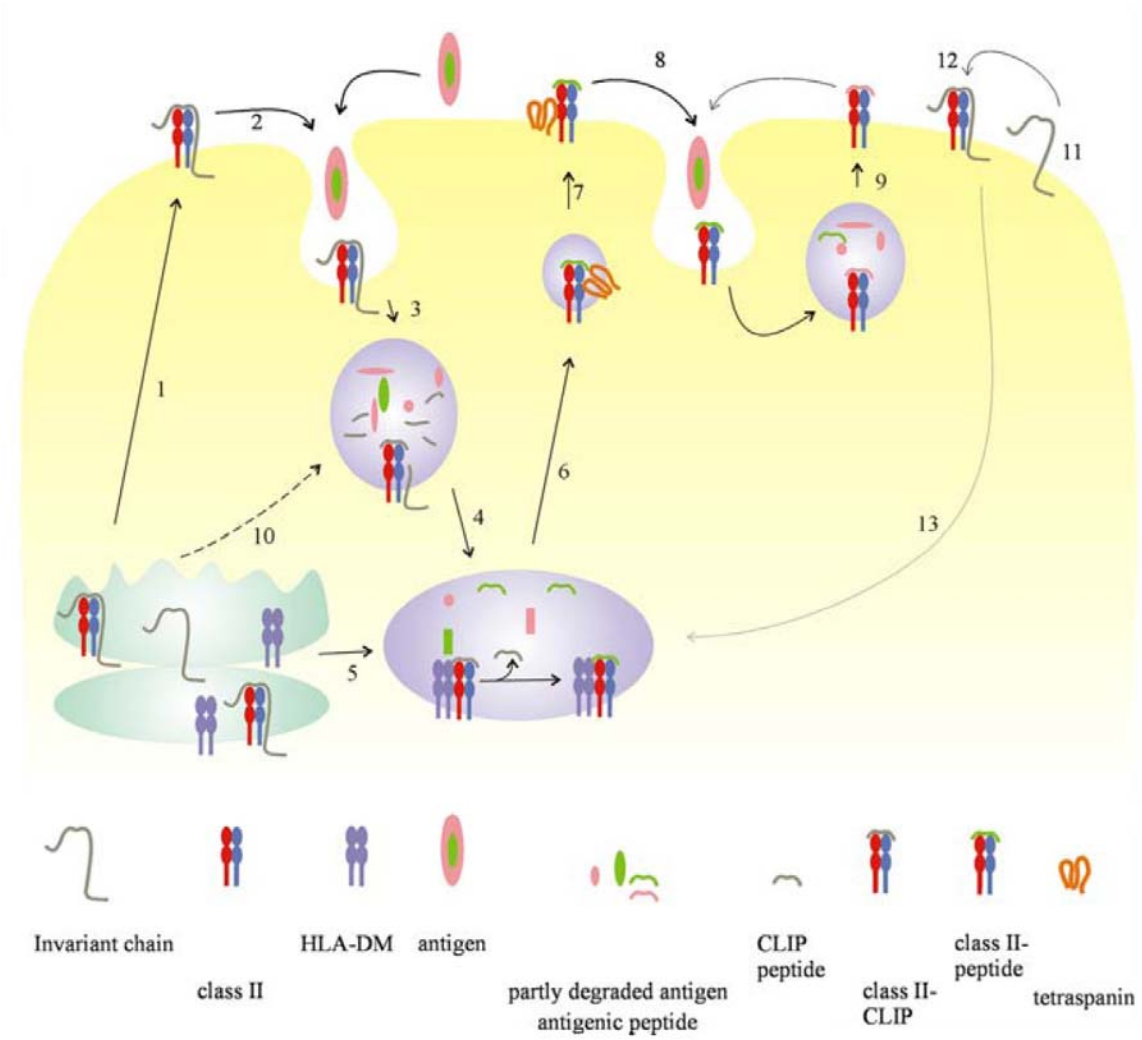

Figure 8. MHC class II/Ii transport route. Obtained from Gregers *et al*. Intracellular Pathogens in Membrane Interactions and Vacuole Biogenesis (2003). 1) The $(\alpha\beta Ii)_3$ complex is transported to the plasma membrane where (2) it is rapidly internalized, and targeted to early endosomes (3) where degradation of Ii begins. The complex is then transported to later endocytic compartments with higher proteolytic activity (4). MHC class II-CLIP associates with HLA-DM, which has been transported directly from ER (5) HLA-DM replaces CLIP with peptides and a conformational change in the MHC class II-peptide complex induces the transport towards (6) and release to the cell surface (7). Mature peptide-MHC class II complexes can recycle at the plasma membrane (8, 9). A minor fraction of MHC class II-Ii complexes may be transported directly to early endosomes (10). Ii is produced in excess of class II molecules, and may be released from ER in the absence of class II (11). These Ii molecules may interact with mature MHC class II molecules at the plasma membrane (12) and thereby target MHC class II to late endosomes or PLC for new rounds of peptide loading (13). See text for details.

describes an indirect transport via the PM[169-171], a third possibility is that transport may occur by both pathways (Figure 8). The cytoplasmic tail of Ii contains two leucine based signals ($_3$DQRDLI$_8$ and $_{12}$EQLPML$_{17}$)[51,172,173]. Both signals are independently sufficient for lysosomal localization of Ii, but L$_7$I$_8$ is a more potent endocytosis motif[174]. Ii has been shown to interact *in vitro* with both AP-1 (mediates sorting between TGN and endosomes)[175] and AP-2 μ-subunits via its di-leucine based signals[63,146]. Recent studies using RNA interference against constitutive subunits of the adaptor complexes indicates that the major $(\alpha\beta Ii)_3$ pathway is via the plasma membrane as depletion of AP-2 lead to a large increase in surface $(\alpha\beta Ii)_3$ whereas depletion of AP-1/-3/-4 had little or no effect[176,177]. Several earlier studies using dominant negative dynamin (K44A)[178] or clathrin[179], selective inactivation of EE[180] and AP-3 deficient cells[181,182] have provided similar results. Other mechanisms for sorting from the TGN exist however[183]. 'Golgi-localized/γ ear-containing/ADP-ribosylation factor-binding' (GGA) proteins bind to motifs that contain a cluster of acidic residues followed by a di-leucine signal[184,185]. Such a cluster is present before the $_3$DQRDLI$_8$ motif in the cytoplasmic tail of Ii, however, experiments performed in our lab show that the different GGAs do not interact with this motif (C. Fladeby *et al.* unpublished data). At the PM $(\alpha\beta Ii)_3$ is rapidly internalized[169,186] and traffics through early endosomes to late endosomes and lysosomes. Along this pathway MHC class II is gradually transferred to intra-lumenal vesicles, and Ii is processed to allow peptides access to the peptide binding groove.

2.3 Ii processing and proteases in endosomal compartments

During the passage through the degradation pathway Ii and internalized antigen are sequentially degraded by an array of specific and non-specific proteases (Figure 9). This processing and the proteases responsible are essential components in MHC class II antigen presentation, and have been extensively studied (reviewed in references[187-189]). However, as there are multiple enzymes present in the class II/antigen pathway, with broad and sometimes overlapping specificities, the question remains whether the generic protease activity is sufficient or whether specific enzymes are required[190]. The first step in Ii processing is the removal of the C-terminal domain. If cysteine proteases are

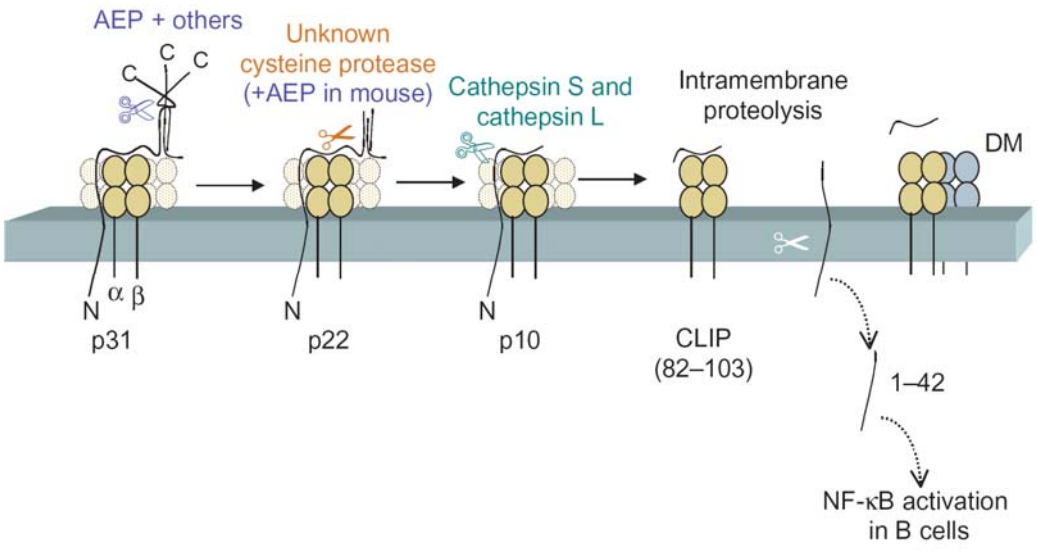

Figure 9. Invariant chain processing. Obtained from Watts, Nat Immunol (2004). Intermediates in processing include p22, p10 and CLIP. DM releases CLIP and, with reduced efficiency, catalyzes release of the p10 intermediate as well. Intramembrane proteolysis of Ii releases an N-terminal fragment that may drive B cell differentiation via NF-κB activation.

inhibited using leupeptin, a 22 kDa fragment of Ii accumulates[191,192] (p22), the enzyme responsible for the initial cleavage under these conditions has been identified as asparagine endopeptidase (AEP). AEP targets N_{155} and possibly N_{132} in the lumenal portion of Ii[193]. Cysteine proteases remove the lumenal trimerization domain generating the 18 kDa and 10 kDa Ii products (p18 and p10 respectively). p22, p18 and p10 all maintain the nonameric structure (and class II interaction)[192,194] through interactions within the TM segment. The next step in Ii processing has been attributed to cathepsin S and inhibition of this enzyme leads to accumulation of the p10 fragment[195] and delayed transport of at least some MHC class II haplotypes[196,197]. Other enzymes may however substitute for cathepsin S, and cathepsin L[198], cathepsin V[199] and cathepsin F[200] have all been shown to process p10 in cells lacking this enzyme. Processing of p10 leaves only CLIP bound to the peptide binding groove of MHC class II, and removal of this is catalyzed by HLA-DM. A final cleavage event may however take place. As shown for murine B cells, an intra-membrane cleavage event (at residues 42-44) releases the cytosolic portion of Ii and this may initiate a signaling cascade resulting in the activation of NF-κB and transcription of target genes[65].

The presence and activity of certain specific proteases is critically important for the optimal generation of some T cell epitopes, whereas other epitopes are susceptible to destructive processing and require the inactivity of certain proteases. Yet other epitopes again may have less specific processing requirements. As previously mentioned, the endocytic pathway becomes gradually more acidic the deeper into it an antigen progresses, and this in itself contributes to regulate the activity of some pH dependent enzymes. Adding to this, certain proteases are specifically regulated at the transcriptional level and also by activating- and inhibiting factors. Interestingly the p41 isoform of Ii is involved in regulating the activity of cathepsin L through the stabilizing action of its 65-residue fragment[189,201-203].

2.4 Retrograde transport of MHC class II molecules

The endocytic pathway has generally been considered a one-way street (with the obvious exception of the sorting compartments/early endosomes/recycling endosomes) whereby if a molecule passes the point of no return, it cannot escape the finality of degradation in lysosomes at the journeys end. However for some molecules, such as CD63[204] and MHC class II[205-207], this does not seem to be the case. The retrograde pathway for class II molecules initially requires the translocation from the inner to the outer membranes of the peptide loading compartments, the mechanisms involved in these "back-fusion" events are however as yet unresolved[208]. After transfer to the limiting membrane, MHC class II is transported to the cell surface. Also here the machinery involved remains elusive, but it seems that the transport may occur both by carrier vesicles[209,210] and tubular extensions[205,206,211]. Both routes are dependent on the cytoskeleton and motor-proteins, and this would require specific adaptors for attachment, tetraspanins could possibly be involved. An alternative retrograde pathway for MHC class II is the fusion of the limiting membrane of peptide loading compartments with the PM and consequent release of class II containing vesicles, exosomes[212]. These secreted particles are able to activate naïve T cells and have been used successfully for *in vivo* immunization for anti-tumor immunity[213].

Retrograde transport of MHC class II in DCs represents a particular paradigm. As mentioned initially, imDCs retain their class II molecules largely internally, whereas after exposure to pathogenic agents or inflammatory stimuli, they up-regulate surface expression of class II (and co-stimulatory molecules). This clearly involves a tight regulation of class II transport, and several mechanisms have been proposed. These include regulating assembly of stable class II-peptide complexes by reduced degradation of Ii (regulating protease activity)[2,214] and modulation of endocytic activity (regulating small GTPases, these studies imply a somewhat different transport pattern with no retrograde transport from lysosomes involved)[5,215]. Adding to this, a recent study has shown that an elevated expression of Ii, as has been reported in imDCs[23], may by itself cause the redistribution of mature, as well as newly synthesized class II, to loading compartments indicating that controlling Ii synthesis will effectively regulate class II plasma membrane expression (Landsverk *et al.* Manuscript), . It seems likely however, that several mechanisms will be operating in parallel.

2.5 MHC class II and Ii at the plasma membrane

MHC class II may be found at the cell surface in at least two distinct forms. As mentioned above, newly synthesized (αβIi)3 complexes are transiently exposed at the PM *en route* to loading compartments, these are however, rapidly internalized and contribute little to the PM expression. In contrast mature, peptide loaded MHC class II may reside stably at the cell surface for several days after activation with pathogenic-/innflammatory stimuli[216]. These molecules may however be internalized to recycling compartments, where they may load or exchange peptides[9], before returning to the cell surface. This internalization is independent on Ii, it is instead mediated by a leucine-based signal located in the MHC class II β chain (LL in mice[47] and a corresponding FL in humans[217]). However, it has recently been shown that these mature class II molecules are also susceptible to the effect of free-Ii transiting the cell surface, and may be redirected also to more acidic compartments (Landsverk *et al.* Manuscript).

MHC class II may partition into specific membrane micro-domains (lipid rafts) at the cell surface[218], and this has been suggested to be of vital importance for activation of naïve T

cells[219]. The association with these lipid rafts may occur before antigen loading and has been proposed to facilitate the enrichment of specific peptide-MHC combinations in these membrane microdomains[220,221]. The existence of lipid rafts is, however, somewhat controversial. Their existence is largely based on the observation that a significant fraction of the membrane is detergent insoluble, and the composition of the isolated rafts is markedly dependent on the conditions used for their preparation[222,223]. Studies using advanced microscopy techniques have so far produced inconclusive and/or contradictory results[223-225], but a recent report indicates that they are not involved in the organization and signalling from the TcR[226]. Lipid raft partitioning has also been implicated in signaling from class II molecules[227], and disruption of raft association by methyl-β-cyclo-dextrin results in inhibition of class II mediated activation of protein tyrosine kinases (PTKs), and cell-cell adhesion[218], but not ERK1/2 activation[218]. Signaling from class II molecules can trigger various signaling pathways and regulate numerous APC activities such as cytokine production, maturation and apoptosis. Ligation and (in some cases) dimerization can result in Src family PTK activation (cytokine production), protein kinase C (PKC) activation (apoptosis) and activation of mitogen-activated protein kinase (MAPK) cascades (reviewed by Al-Daccak[228]). Signaling from class II molecules may also be involved in regulating class II transport, and Boes *et al.* have shown that after an initial encounter with T cells, DCs extend MHC class II dense tubules toward the T cell-DC interface[205]. Tetraspanins have also been implicated in organizing class II and accessory molecules in specific membrane micro-domains. Interestingly, these domains were found to be highly enriched in specific peptide-MHC combinations, and their disruption severely impaired T cell activation[229]. The successful activation of a naïve T cell may require as few as 10 specific MHC class II-peptide combinations[230]. This represents less than 0,1% of the total amount of class II on an APC surface, and considering the number of peptide-MHC combinations present, a mechanism for sequestering specific combinations would be highly convenient.

Aims of the project

The influence of Invariant chain on the traffic of MHC class II molecules is, in the textbooks and current reviews, restricted to the initial transport of newly synthesized MHC class II to from ER to the peptide loading compartments. However, the elevated expression of Ii compared to class II has prompted two groups to suggest a potential post ER interaction between MHC class II and Ii. In 1995 Henne *et al.* proposed that free-Ii could associate with empty class II molecules at the PM and promote their endocytosis and delivery to loading compartments[231], and this was later elaborated on by Moldenhauer *et al.*[231]. In 1999 Triantafilou *et al.*[232,233] detected $(\alpha\beta Ii)_3$ complexes at the cell surface in their studies and came with a similar proposition. However, these studies were not able to distinguish between newly synthesized $(\alpha\beta Ii)_3$ complexes assembled in the ER and free-Ii associating with class II at later time points. The post-ER interaction therefore remained highly hypothetical, and has not been implicated in MHC class II trafficking. With the rapid development of new life-sciences tools it has now become possible to distinguish between the two. This study was set up to solve this question.

The aims of the study:

a) Definitively establish a post-ER interaction between Ii and MHC class II
b) Examine the consequences of this interaction on MHC class II distribution
c) Determine the domains of Ii required for plasma membrane clearance of class II

To address these questions we utilized a photoactivable green fluorescent protein (PAGFP), fused to MHC class II, in combination with an inducible expression system for Ii. This allowed us to specifically image the fraction of class II available at photo-activation and their distribution under the influence of newly induced Ii. Molecular cloning techniques were used to generate chimeric proteins and deletion mutants. Biochemical methods were applied to obtain quantitative data, and scanning laser confocal microscopy to visualize protein trafficking in real-time.

3.0 The photoactivable green fluorescent protein

The photoactivable green fluorescent protein (PAGFP) was initially presented by Patterson and Lippincott-Schwartz in science 2002[234], it is a derivative of wtGFP that remains 'in-excitable' to the 488 nm laser until 'photo-converted' with a 405 nm laser (Figure 10). This makes it a convenient tool for imaging a sub-population, in time or space, of 'PAGFP-tagged' molecules[235].

Studies by other groups have previously used fluorescent proteins attached to the human or mouse MHC class II β chain[103,205,236] and we attempted to do the same with PAGFP. We constructed fusion proteins using two different HLA-DRβ alleles with various linker sequences. However, all fusion constructs were retained in the ER, whereas the EGFP constructs made in parallel trafficked normally.

PAGFP is in sequence similar to wtGFP, the only difference between the two is a threonine to histidine substitution in the 203 position (T203H). The enhanced GFP

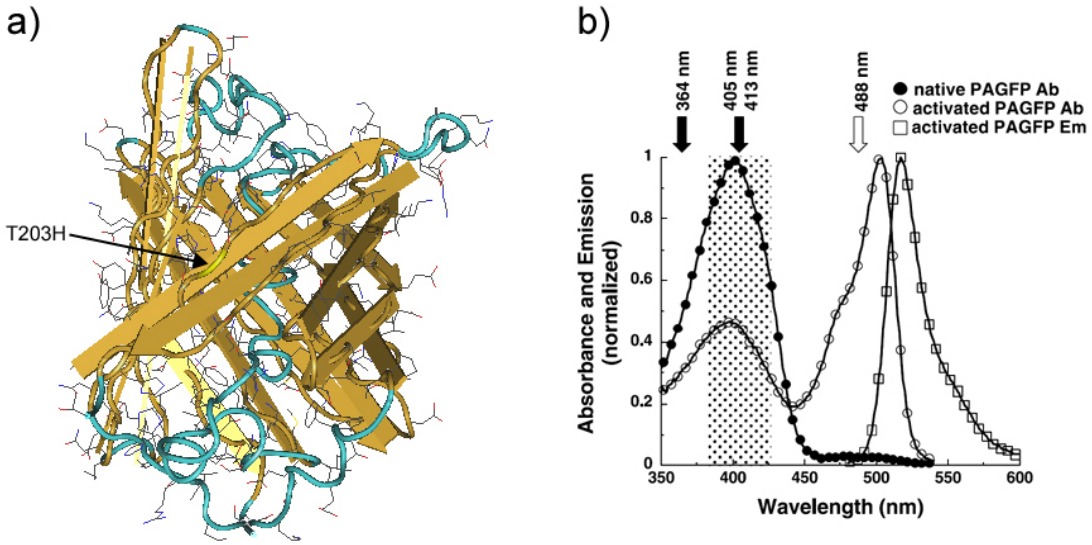

Figure 10. wtGFP β-barrel and PAGFP exitation/emission spectra and photoactivation. a) Adapted from Van Thor *et al*. (2001) and b) obtained from Patterson and Lippincott-Schwartz, Methods (2004). a) Wild type GFP β-barrel. The replaced threonine indicated by arrow. b) PAGFP absorbance and emission spectra. Native PA-GFP absorbance spectrum is represented by the solid circles. Photoactivated PAGFP absorbance spectrum is shown in open circles and emission spectrum in open squares.

(EGFP) is slightly further removed from PAGFP, with an additional serine to threonine substitution in position 65 (S65T). This small difference is however enough to abolish the effect of the T203H mutation. The T203H substitution, rendering our fusion constructs incapable of export from the ER, comes on the outer surface of the GFP beta barrel (Figure 10), presumably causing some minor conformational change in GFP. This might interfere with the proper dimerization of the α and β chains, and consequent sequestration in the ER. However, attaching PAGFP to the α chain of the heterodimer, resulted in proper dimerization and consequent transport out of the ER.

3.1 The metallothionein inducible invariant chain

The pMEP4 vector from Invitrogen contains the human metallothionein (MT) promoter hMTIIA[237]. The Metal response element-binding transcription factor-1(MTF-1) binds specifically to metal response elements (MREs) and trans-activates MT gene expression in response to zinc and cadmium[238]. MTF-1 is highly conserved in evolution, mouse and human MTF-1 are 93% similar, and mouse MTF-1 can activate the human promoter[239]. Transfected into MDCK cells hMTIIA is effectively activated by addition of cadmium, indicating that also the canine MTF-1 is of high homology.

The inducible expression system containing the metallothionein promoter has the major advantage of being fast and easy to use. As opposed to other transfection systems it requires only one transfection, and not the multiple rounds needed in other systems. It is however, somewhat prone to leakage, with a varying degree of low basal Ii expression. This does not constitute a direct problem in the study presented. We have all the same constantly compared our results with un-induced cells and cells that are induced but lacking Ii (see for instance figure 2B in manuscript). A very high basal level would of course be detrimental to our studies, and although this is very rarely the case, most imaging was conducted with the monoclonal anti-Ii antibody BU45 conjugated to alexa 546 in the medium. This has the additional benefit of verifying a high Ii expression as cause for the observed effect. Cadmium will also turn on a set of metallothionein inducible genes[240]. However, in the study presented here, we could detect no interference with the MHC class II/Ii transport as a consequence of these endogenous genes being

turned on. The trafficking and interactions observed were the same in the absence of $CdCl_2$. Cadmium is cytotoxic to cells if administered in high doses. Titration studies have previously been carried out in our lab to assess growth inhibition under varying doses. We have on the basis of these used 15-25μM $CdCl_2$ in the experiments, this dosage provides adequate induction without detectable growth inhibition.

3.2 Cross-species considerations

In the presented study we have, mostly in parallel, used both human and murine class II molecules, in combination with exclusively human Ii. The murine and human Ii are however, highly homologous (76.2% identical/85.1% positives)[1] and mouse I-Ak effectively co-immunoprecipitates human Ii[241,242]. I-Ak and HLA-DR1 are structurally similar, display large sequence homology (52.3%/64.5% for α, 61.8%/70% for β)[1] and almost identical organization of the conserved domains and sequences[2]. It has been previously shown that transport and function proceeds similarly with both combinations (HLA-DR/Ii or I-Ak/Ii)[243]. Another aspect that must be considered is the use of human Ii in a canine cell line. As for mouse Ii, the predicted canine Ii[3] is highly similar to human. The N-terminal region, which interacts with the essential trafficking machinery, is aligned with human Ii in figure 11. As indicated, the charge distribution and sorting signals are conserved, implying that the interactions described above apply also in this species.

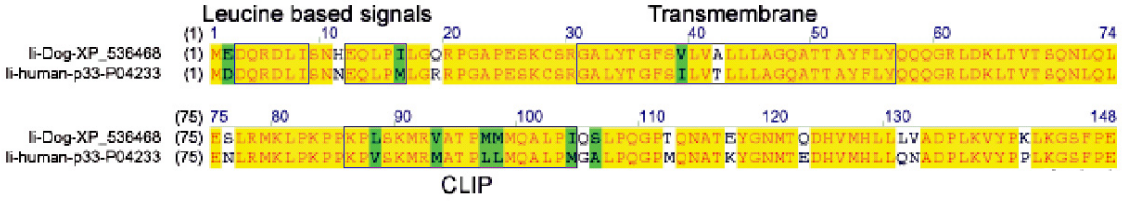

Figure 11. Comparizon of human and canine Ii N-terminal sequences. Sequences acquired from NCBI, (accession numbers indicated) and aligned using alignX, VNTI (InforMax, Invitrogen).

[1] Calculated using alignX, mouse Ii (P04233)/human Ii (P04233-N-terminal extension), H-2Akα (P01910)/ HLA-DR1α (P01903), H-2Akβ (P06343)/HLA-DR1β (P04229)
[2] Determined by analysis of domain architecture of sequences above in Vector NTI, and their 3 dimensional structures (I-Ak by Fremont et al 1997, HLA-DR1 by Zavala-Ruiz *et al*. 2003)
[3] LOC479329 on chromosome 4

3.3 Cell lines

MelJuSo is a human melanoma cell line that expresses endogenous HLA-DR3. It was used in this study to examine the various HLA-DR_{PAGFP} constructs. Single MHC class II α or β chains are retained in the ER, and the same applies to the early HLA-DR_{PAGFP} constructs, it was therefore convenient to use a cell line that expressed both α and β and would assemble heterodimers and transport the constructs to the plasma membrane.
Madin Darby canine kidney (MDCK) cells are from a female cocker spaniel[244]. There are two variants, MDCK I and MDCK II, the latter being the original strain and the one used in the study presented here. MDCK cells are easy to grow, highly resilient and easily manipulated, they form polarized mono-layers on almost any substrate making them convenient for microscopy studies. When confluent, MDCK cells sort their class II molecules to the basolateral side and as their tight-junctions are close to the apical face, this makes it easy to get a nice confocal slice with clear cell surface fluorescence.
M1 is a human fibroblast cell line derived from the SV40-transformed xeroderma pigmentosum cell line SV40XP12RO[245]. It was used for some of the immuno-fluorescence microscopy mentioned in the manuscript, but is reportedly highly sensitive to ultraviolet light, which makes it an unsuitable recipient for PAGFP.

3.4 Stable transfection

PAGFP is ideally suited to our approach as it is entirely invisible before activation (Figure 12). This however, makes it impossible to detect positive cells without activating them, and time consuming to find transfected cells when doing transient transfections. Added to this there is the requirement for the cells not only to be expressing HLA-DR1α-PAGFP, but also HLA-DR3β and the inducible Ii. All together this makes it difficult, if not impossible, to conduct these experiments on transiently transfected cells. We therefore generated cell-lines expressing HLA-DR1α-PAGFP and HLA-DR3β with or without Ii. Stably transfected cells are not, however, as stable as the given term implies, and individual cells within the population are prone to discard one or two, or all constructs. The reason for this is not well understood, but non-homologous

recombination usually results in the insertion of multiple copies of the transfected construct(s) (tandem repeats). It is therefore likely that during mitosis there will be a high frequency of crossing over and consequent 'breakage-fusion-bridge' cycles[246], which may eventually lead to expulsion of the transfected sequences from the genome. The frequency of these events seems to increase with the number of constructs inserted, which is not surprising considering the similarity of carrier vectors, consequently what started as the 'perfect' cell line may degenerate into something rather less ideal.

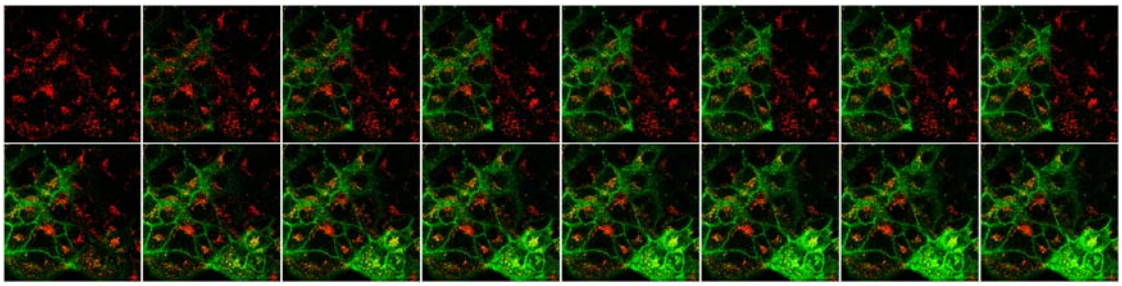

Figure 12. Photoactivation. Left half of micrograph photoactivated (405 nm laser at 40%) in the upper sequence, right half in the lower sequence. Cells are $MDCK_{Ii+\beta\alpha PA}$ imaged with 488 nm at 6%, 543 nm at 2% (lysotracker-red), 320x320 pixels, 1.4xZoom, 60/1.1 oil immersion objective, on an Olympus FV1000 scanning laser confocal microscope.

3.5 Live scanning laser confocal imaging

The Olympus FV1000 confocal scanning laser microscope is the perfect companion to the PAGFP. With its real-time bleach capacity it is possible to photoactivate and image at the same time (Figure 12). This is highly convenient for assessing amount of 405nm laser needed for activation as too much laser will bleach the fluorescent probes. The sequential line-by-line excitation/acquisition minimizes 'bleed-through' and provides reliable fluorochrome localization/colocalization. PAGFP emits significantly less light than EGFP under the same conditions (laser intensity), thus it requires more laser, a higher expression level or more enhancement of the signal. Higher laser intensity is unfortunate as it will bleach the sample, it may also cause damage to the cells (photocytoxity). A very high expression level is not desirable as it might cause stress and other bi-effects in the cells. Enhancing the signal increases the noise to signal ratio, making the images less clear. We have chosen to image cells with an intermediate PAGFP expression level with

a slightly higher 488 nm laser intensity (3-9%), and an intermediate to high noise to signal ratio. Transmission light images were obtained throughout the experiments to verify that the cells remained healthy. This allows us to obtain the required information, the images, however, are not always of the highest resolution. As mentioned above, stably transfected cells have a tendency to expel one or more of the constructs, and consequently we have imaged at low zoom to observe many cells and used internalization of BU45 as an Ii-expression positive marker. All live-imaging experiments have been repeated at least three times, the results in the manuscript and supplementary movies are of representative cells in a single experiment.

1. Janeway, C. A., Travers P., Walport M., Shlomchik M. J. Immunobiology; the immune system in health and disease. *Garland Science Publishing* **6th Edition** (2005).
2. Trombetta, E. S. & Mellman, I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol* **23**, 975-1028 (2005).
3. Ardavin, C. Origin, precursors and differentiation of mouse dendritic cells. *Nat Rev Immunol* **3**, 582-90 (2003).
4. Wilson, N. S. & Villadangos, J. A. Lymphoid organ dendritic cells: beyond the Langerhans cells paradigm. *Immunol Cell Biol* **82**, 91-8 (2004).
5. Wilson, N. S. & Villadangos, J. A. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. *Adv Immunol* **86**, 241-305 (2005).
6. Pulendran, B. Variegation of the immune response with dendritic cells and pathogen recognition receptors. *J Immunol* **174**, 2457-65 (2005).
7. de Jong, E. C., Smits, H. H. & Kapsenberg, M. L. Dendritic cell-mediated T cell polarization. *Springer Semin Immunopathol* **26**, 289-307 (2005).
8. Gregers, T. F., Nordeng T. W., Bakke O. Antigen Presentation by MHC Class II Molecules. *Intracellular Pathogens in Membrane Interactions and Vacuole Biogenesis* (2003).
9. Pinet, V. M. & Long, E. O. Peptide loading onto recycling HLA-DR molecules occurs in early endosomes. *Eur J Immunol* **28**, 799-804 (1998).
10. Neefjes, J. CIIV, MIIC and other compartments for MHC class II loading. *Eur.J.Immunol.* **29**, 1421-1425 (1999).
11. Brocke, P., Garbi, N., Momburg, F. & Hammerling, G. J. HLA-DM, HLA-DO and tapasin: functional similarities and differences. *Curr Opin Immunol* **14**, 22-9 (2002).
12. Horton, R. et al. Gene map of the extended human MHC. *Nat Rev Genet* **5**, 889-99 (2004).
13. Gorer, P. A. The detection of a hereditary antigenic difference in the blood of mice by means of human group A serum. *J. Genet.* **32**, 17-31 (1936).
14. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature* **401**, 921-3 (1999).
15. Mungall, A. J. et al. The DNA sequence and analysis of human chromosome 6. *Nature* **425**, 805-11 (2003).
16. Mach, B., Steimle, V., Martinez-Soria, E. & Reith, W. Regulation of MHC class II genes: lessons from a disease. *Annu Rev Immunol* **14**, 301-31 (1996).
17. Shewey, L. M., Beaty, J. S., Andersen, L. C. & Nepom, G. T. Differential expression of related HLA class II DQ beta genes caused by nucleotide variation in transcriptional regulatory elements. *J Immunol* **148**, 1265-73 (1992).
18. Louis, P., Vincent, R., Cavadore, P., Clot, J. & Eliaou, J. F. Differential transcriptional activities of HLA-DR genes in the various haplotypes. *J Immunol* **153**, 5059-67 (1994).
19. Peijnenburg, A. et al. Discoordinate expression of invariant chain and MHC class II genes in class II transactivator-transfected fibroblasts defective for RFX5. *J Immunol* **163**, 794-801 (1999).

20. Machamer, C. E. & Cresswell, P. Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J Immunol* **129**, 2564-9 (1982).
21. Kvist, S., Wiman, K., Claesson, L., Peterson, P. A. & Dobberstein, B. Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell* **29**, 61-9 (1982).
22. Nguyen, Q. V. & Humphreys, R. E. Time course of intracellular associations, processing, and cleavages of Ii forms and class II major histocompatibility complex molecules. *J Biol Chem* **264**, 1631-7 (1989).
23. Kampgen, E. et al. Class II major histocompatibility complex molecules of murine dendritic cells: synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture. *Proc Natl Acad Sci U S A* **88**, 3014-8 (1991).
24. Reith, W. et al. Congenital immunodeficiency with a regulatory defect in MHC class II gene expression lacks a specific HLA-DR promoter binding protein, RF-X. *Cell* **53**, 897-906 (1988).
25. Reith, W., Siegrist, C. A., Durand, B., Barras, E. & Mach, B. Function of major histocompatibility complex class II promoters requires cooperative binding between factors RFX and NF-Y. *Proc Natl Acad Sci U S A* **91**, 554-8 (1994).
26. Moreno, C. S., Beresford, G. W., Louis-Plence, P., Morris, A. C. & Boss, J. M. CREB regulates MHC class II expression in a CIITA-dependent manner. *Immunity* **10**, 143-51 (1999).
27. Reith, W. et al. Cooperative binding between factors RFX and X2bp to the X and X2 boxes of MHC class II promoters. *J Biol Chem* **269**, 20020-5 (1994).
28. Masternak, K. et al. CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhanceosome complex. *Genes Dev* **14**, 1156-66 (2000).
29. LeibundGut-Landmann, S. et al. Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *Eur J Immunol* **34**, 1513-25 (2004).
30. Ting, J. P. & Trowsdale, J. Genetic control of MHC class II expression. *Cell* **109 Suppl**, S21-33 (2002).
31. Westerheide, S. D., Louis-Plence, P., Ping, D., He, X. F. & Boss, J. M. HLA-DMA and HLA-DMB gene expression functions through the conserved S-X-Y region. *J Immunol* **158**, 4812-21 (1997).
32. Taxman, D. J., Cressman, D. E. & Ting, J. P. Identification of class II transcriptional activator-induced genes by representational difference analysis: discoordinate regulation of the DN alpha/DO beta heterodimer. *J Immunol* **165**, 1410-6 (2000).
33. Nagarajan, U. M. et al. Class II transactivator is required for maximal expression of HLA-DOB in B cells. *J Immunol* **168**, 1780-6 (2002).
34. Khalil, H. et al. Class II transactivator-induced expression of HLA-DO(beta) in HeLa cells. *Tissue Antigens* **60**, 372-82 (2002).
35. Wright, K. L. et al. CIITA stimulation of transcription factor binding to major histocompatibility complex class II and associated promoters in vivo. *Proc Natl Acad Sci U S A* **95**, 6267-72 (1998).

36. Zika, E. & Ting, J. P. Epigenetic control of MHC-II: interplay between CIITA and histone-modifying enzymes. *Curr Opin Immunol* **17**, 58-64 (2005).
37. Muhlethaler-Mottet, A., Otten, L. A., Steimle, V. & Mach, B. Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *Embo J* **16**, 2851-60 (1997).
38. van den Elsen, P. J., Holling, T. M., Kuipers, H. F. & van der Stoep, N. Transcriptional regulation of antigen presentation. *Curr Opin Immunol* **16**, 67-75 (2004).
39. Kuchtey, J., Pennini, M., Pai, R. K. & Harding, C. V. CpG DNA induces a class II transactivator-independent increase in class II MHC by stabilizing class II MHC mRNA in B lymphocytes. *J Immunol* **171**, 2320-5 (2003).
40. Shih, N. Y., Soesilo, I. & Floyd-Smith, G. Stabilization of invariant chain mRNA by 12-O-tetradecanoylphorbol-13-acetate is blocked by IFN-gamma in a murine B lymphoma cell line. *J Interferon Cytokine Res* **17**, 747-55 (1997).
41. Kaufman, J. F., Auffray, C., Korman, A. J., Shackelford, D. A. & Strominger, J. The class II molecules of the human and murine major histocompatibility complex. *Cell* **36**, 1-13 (1984).
42. Kjær-Nielsen, L., Perera, J. D., Boyd, L. F., Margulies, D. H. & McCluskey, J. The extracellular domains of MHC class II molecules determine their processing requirements for antigen presentation. *J.Immunol.* **144**, 2915-2924 (1990).
43. Wettstein, D. A., Boniface, J. J., Reay, P. A., Schild, H. & Davis, M. M. Expression of a class II major histocompatibility complex (MHC) heterodimer in a lipid-linked form with enhanced peptide/soluble MHC complex formation at low pH. *J.Exp.Med.* **174**, 219-228 (1991).
44. Cosson, P. & Bonifacino, J. S. Role of transmembrane domain interactions in the assembly of class II MHC molecules. *Science* **258**, 659-662 (1992).
45. Brown, J. H. et al. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364**, 33-9 (1993).
46. Fremont, D. H., Monnaie, D., Nelson, C. A., Hendrickson, W. A. & Unanue, E. R. Crystal structure of I-Ak in complex with a dominant epitope of lysozyme. *Immunity* **8**, 305-17 (1998).
47. Zhong, G., Romagnoli, P. & Germain, R. N. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. *J Exp Med* **185**, 429-38 (1997).
48. Simonsen, A. et al. Polarized transport of MHC class II molecules in Madin-Darby canine kidney cells is directed by a leucine-based signal in the cytoplasmic tail of the beta-chain. *J Immunol* **163**, 2540-8 (1999).
49. Pinet, V., Vergelli, M., Martin, R., Bakke, O. & Long, E. O. Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature* **375**, 603-6 (1995).
50. Anderson, M. S. & Miller, J. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc Natl Acad Sci U S A* **89**, 2282-6 (1992).

51. Odorizzi, C. G. et al. Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *J Cell Biol* **126**, 317-30 (1994).
52. O'Sullivan, D. M., Noonan, D. & Quaranta, V. Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. *J Exp Med* **166**, 444-60 (1987).
53. Strubin, M., Berte, C. & Mach, B. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *Embo J* **5**, 3483-8 (1986).
54. Lotteau, V. et al. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348**, 600-605 (1990).
55. Schutze, M.-P., Peterson, P. A. & Jackson, M. R. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO J.* **13**, 1696-1705 (1994).
56. Khalil, H. et al. The MHC class II beta chain cytoplasmic tail overcomes the invariant chain p35-encoded endoplasmic reticulum retention signal. *Int Immunol* **15**, 1249-63 (2003).
57. Koch, N. Posttranslational modifications of the Ia-associated invariant protein p41 after gene transfer. *Biochemistry* **27**, 4097-4102 (1988).
58. Cresswell, P. Assembly, transport, and function of MHC class II molecules. *Annu Rev Immunol* **12**, 259-93 (1994).
59. Pieters, J. MHC class II-restricted antigen processing and presentation. *Adv Immunol* **75**, 159-208 (2000).
60. Ashman, J. B. & Miller, J. A role for the transmembrane domain in the trimerization of the MHC class II-associated invariant chain. *J.Immunol.* **163**, 2704-2712 (1999).
61. Gedde-Dahl, M. et al. Exon 6 is essential for invariant chain trimerization and induction of large endosomal structures. *J.Biol.Chem.* **272**, 8281-8287 (1997).
62. Bijlmakers, M. E., Benaroch, P. & Ploegh, H. L. Mapping functional regions in the lumenal domain of the class II-associated invariant chain. *J.Exp.Med.* **180**, 623-629 (1994).
63. Hofmann, M. W. et al. The Leucine-based Sorting Motifs in the Cytoplasmic Domain of the Invariant Chain Are Recognized by the Clathrin Adaptors AP1 and AP2 and their Medium Chains. *J. Biol. Chem.* **274**, 36153-36158 (1999).
64. Bertolino, P. et al. Deletion of a C-terminal sequence of the class II-associated invariant chain abrogates invariant chains oligomer formation and class II antigen presentation. *J Immunol* **154**, 5620-9 (1995).
65. Matza, D., Kerem, A. & Shachar, I. Invariant chain, a chain of command. *Trends Immunol* **24**, 264-8 (2003).
66. Koonce, C. H. & Bikoff, E. K. Dissecting MHC class II export, B cell maturation, and DM stability defects in invariant chain mutant mice. *J Immunol* **173**, 3271-80 (2004).
67. Leng, L. et al. MIF signal transduction initiated by binding to CD74. *J Exp Med* **197**, 1467-76 (2003).

68. Romagnoli, P. & Germain, R. N. The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J.Exp.Med.* **180**, 1107-1113 (1994).
69. Ghosh, P., Amaya, M., Mellins, E. & Wiley, D. C. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* **378**, 457-462 (1995).
70. Gautam, A. M., Pearson, C., Quinn, V., McDevitt, H. O. & Milburn, P. J. Binding of an invariant-chain peptide, CLIP, to I-A major histocompatibility complex class II molecules. *Proc Natl Acad Sci U S A* **92**, 335-9 (1995).
71. Malcherek, G., Gnau, V., Jung, G., Rammensee, H. G. & Melms, A. Supermotifs enable natural invariant chain-derived peptides to interact with many major histocompatibility complex-class II molecules. *J Exp Med* **181**, 527-36 (1995).
72. Sette, A., Southwood, S., Miller, J. & Appella, E. Binding of major histocompatibility complex class II to the invariant chain-derived peptide, CLIP, is regulated by allelic polymorphism in class II. *J.Exp.Med.* **181**, 677-683 (1995).
73. Lee, C. z. & McConnell, H. M. A general model of invariant chain association with class II major histocompatibility complex proteins. *Proc.Natl.Acad.Sci.USAUSA* **92**, 8269-8273 (1995).
74. Kropshofer, H., Vogt, A. B. & Hämmerling, G. J. Structural features of the invariant chain fragment CLIP controlling rapid release from HLA-DR molecules and inhibition of peptide binding. *Proc.Natl.Acad.Sci.USAUSA* **92**, 8313-8317 (1995).
75. Kropshofer, H., Vogt, A. B., Stern, L. J. & Hämmerling, G. J. Self-release of CLIP in peptide loading of HLA-DR molecules. *Science* **270**, 1357-1359 (1995).
76. Wilson, N. A. et al. Invariant chain can bind MHC class II at a site other than the peptide binding groove. *J Immunol* **161**, 4777-84 (1998).
77. Thayer, W. P., Ignatowicz, L., Weber, D. A. & Jensen, P. E. Class II-associated invariant chain peptide-independent binding of invariant chain to class II MHC molecules. *J Immunol* **162**, 1502-9 (1999).
78. Lang, M. L. et al. Mutations in specific I-A(k) alpha(2) and beta(2) domain residues affect surface expression. *Int Immunol* **12**, 777-86 (2000).
79. Castellino, F., Han, R. & Germain, R. N. The transmembrane segment of invariant chain mediates binding to MHC class II molecules in a CLIP-independent manner. *Eur J Immunol* **31**, 841-50 (2001).
80. Roche, P. A., Marks, M. S. & Cresswell, P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* **354**, 392-394 (1991).
81. Bikoff, E. K., Germain, R. N. & Robertson, E. J. Allelic differences affecting invariant chain dependency of MHC class II subunit assembly. *Immunity* **2**, 301-10 (1995).
82. Lamb, C. A. & Cresswell, P. Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *J.Immunol.* **148**, 3478-3482 (1992).
83. Anderson, K. S. & Cresswell, P. A role for calnexin (IP90) in the assembly of class II MHC molecules. *Embo J* **13**, 675-82 (1994).

84. Anderson, K. S. & Cresswell, P. A role for calnexin (IP90) in the assembly of class II MHC molecules. *EMBO J.* **13 (3)**, 675-682 (1994).
85. Schreiber, K. L. et al. Class II histocompatibility molecules associate with calnexin during assembly in the endoplasmic reticulum. *Int Immunol* **6**, 101-11 (1994).
86. Bonnerot, C. et al. Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain. *Embo J* **13**, 934-44 (1994).
87. Lee, M. C., Miller, E. A., Goldberg, J., Orci, L. & Schekman, R. Bi-directional protein transport between the ER and Golgi. *Annu Rev Cell Dev Biol* **20**, 87-123 (2004).
88. Koch, N. & Hammerling, G. J. Ia-associated invariant chain is fatty acylated before addition of sialic acid. *Biochemistry* **24**, 6185-6190 (1985).
89. Sant, A. J., Zacheis, M., Rumbarger, T., Giacoletto, K. S. & Schwartz, B. D. Human Ia alpha- and beta-chains are sulfated. *J.Immunol.* **140**, 155-160 (1988).
90. Spiro, R. C. & Quaranta, V. The invariant chain is a phosphorylated subunit of class II molecules. *J.Immunol.* **143**, 2589-2594 (1989).
91. Elliott, E. A. et al. The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. *J.Exp.Med.* **179**, 681-694 (1994).
92. Pieters, J., Bakke, O. & Dobberstein, B. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J Cell Sci* **106 (Pt 3)**, 831-46 (1993).
93. Alfonso, C. & Karlsson, L. Nonclassical MHC class II molecules. *Annu Rev Immunol* **18**, 113-42 (2000).
94. Lindstedt, R., Liljedahl, M., Peleraux, A., Peterson, P. A. & Karlsson, L. The MHC class II molecule H2-M is targeted to an endosomal compartment by a tyrosine-based targeting motif. *Immunity* **3**, 561-72 (1995).
95. Marks, M. S. et al. A lysosomal targeting signal in the cytoplasmic tail of the beta chain directs HLA-DM to MHC class II compartments. *J Cell Biol* **131**, 351-69 (1995).
96. Copier, J. et al. Targeting signal and subcellular compartments involved in the intracellular trafficking of HLA-DMB. *J Immunol* **157**, 1017-27 (1996).
97. Denzin, L. K. & Cresswell, P. HLA-DM induces CLIP dissociation from MHC class II alphabeta dimers and facilitates peptide loading. *Cell* **82**, 155-165 (1995).
98. Sloan, V. S. et al. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* **375**, 802-806 (1995).
99. Sherman, M. A., Weber, D. A. & Jensen, P. E. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* **3**, 197-205 (1995).
100. van Ham, S. M. et al. Human histocompatibility leukocyte antigen (HLA)-DM edits peptides presented by HLA-DR according to their ligand binding motifs. *J Exp Med* **184**, 2019-24 (1996).
101. Sanderson, F., Thomas, C., Neefjes, J. & Trowsdale, J. Association between HLA-DM and HLA-DR in vivo. *Immunity* **4**, 87-96 (1996).

102. Vogt, A. B., Arndt, S. O., Hammerling, G. J. & Kropshofer, H. Quality control of MHC class II associated peptides by HLA-DM/H2-M. *Semin Immunol* **11**, 391-403 (1999).
103. Zwart, W. et al. Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escape. *Immunity* **22**, 221-33 (2005).
104. Van Ham, S. M. et al. HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr.Biol.* **7**, 950-957 (1997).
105. Liljedahl, M. et al. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *Embo J* **15**, 4817-24 (1996).
106. Denzin, L. K., Sant'Angelo, D. B., Hammond, C., Surman, M. J. & Cresswell, P. Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science* **278**, 106-9 (1997).
107. van Lith, M. et al. Regulation of MHC class II antigen presentation by sorting of recycling HLA-DM/DO and class II within the multivesicular body. *J Immunol* **167**, 884-92 (2001).
108. Douek, D. C. & Altmann, D. M. HLA-DO is an intracellular class II molecule with distinctive thymic expression. *Int Immunol* **9**, 355-64 (1997).
109. Samaan, A. et al. Cellular distribution of a mixed MHC class II heterodimer between DRalpha and a chimeric DObeta chain. *Int Immunol* **11**, 99-111 (1999).
110. Kropshofer, H. et al. A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. *EMBO J.* **17**, 2971-2981 (1998).
111. van Ham, M. et al. Modulation of the major histocompatibility complex class II-associated peptide repertoire by human histocompatibility leukocyte antigen (HLA)-DO. *J Exp Med* **191**, 1127-36 (2000).
112. Liljedahl, M. et al. Altered antigen presentation in mice lacking H2-O. *Immunity* **8**, 233-243 (1998).
113. Hemler, M. E. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu Rev Cell Dev Biol* **19**, 397-422 (2003).
114. Escola, J. M. et al. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* **273**, 20121-7 (1998).
115. Hammond, C. et al. The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J Immunol* **161**, 3282-91 (1998).
116. Schick, M. R. & Levy, S. The TAPA-1 molecule is associated on the surface of B cells with HLA-DR molecules. *J Immunol* **151**, 4090-7 (1993).
117. Levy, S. & Shoham, T. The tetraspanin web modulates immune-signalling complexes. *Nat Rev Immunol* **5**, 136-48 (2005).
118. Stuart, L. M. & Ezekowitz, R. A. Phagocytosis: elegant complexity. *Immunity* **22**, 539-50 (2005).
119. Greenberg, S. & Grinstein, S. Phagocytosis and innate immunity. *Curr Opin Immunol* **14**, 136-45 (2002).
120. Underhill, D. M. & Ozinsky, A. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* **20**, 825-52 (2002).

121. Sallusto, F., Cella, M., Danieli, C. & Lanzavecchia, A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products [see comments]. *J Exp Med* **182**, 389-400 (1995).
122. West, M. A., Prescott, A. R., Eskelinen, E. L., Ridley, A. J. & Watts, C. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol* **10**, 839-48 (2000).
123. Garrett, W. S. et al. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* **102**, 325-34 (2000).
124. Araki, N., Johnson, M. T. & Swanson, J. A. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* **135**, 1249-60 (1996).
125. Sun, P. et al. Small GTPase Rah/Rab34 is associated with membrane ruffles and macropinosomes and promotes macropinosome formation. *J Biol Chem* **278**, 4063-71 (2003).
126. Hamasaki, M., Araki, N. & Hatae, T. Association of early endosomal autoantigen 1 with macropinocytosis in EGF-stimulated A431 cells. *Anat Rec A Discov Mol Cell Evol Biol* **277**, 298-306 (2004).
127. Racoosin, E. L. & Swanson, J. A. M-CSF-induced macropinocytosis increases solute endocytosis but not receptor-mediated endocytosis in mouse macrophages. *J Cell Sci* **102 (Pt 4)**, 867-80 (1992).
128. Norbury, C. C., Hewlett, L. J., Prescott, A. R., Shastri, N. & Watts, C. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* **3**, 783-91 (1995).
129. Harris, J., Werling, D., Hope, J. C., Taylor, G. & Howard, C. J. Caveolae and caveolin in immune cells: distribution and functions. *Trends Immunol* **23**, 158-64 (2002).
130. Oh, P., McIntosh, D. P. & Schnitzer, J. E. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J Cell Biol* **141**, 101-14 (1998).
131. Shajahan, A. N., Tiruppathi, C., Smrcka, A. V., Malik, A. B. & Minshall, R. D. Gbetagamma activation of Src induces caveolae-mediated endocytosis in endothelial cells. *J Biol Chem* **279**, 48055-62 (2004).
132. Shajahan, A. N. et al. Role of Src-induced dynamin-2 phosphorylation in caveolae-mediated endocytosis in endothelial cells. *J Biol Chem* **279**, 20392-400 (2004).
133. Pelkmans, L., Burli, T., Zerial, M. & Helenius, A. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell* **118**, 767-80 (2004).
134. Lanzavecchia, A. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu Rev Immunol* **8**, 773-93 (1990).
135. Bonifaz, L. C. et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* **199**, 815-24 (2004).

136. Bonifaz, L. et al. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* **196**, 1627-38 (2002).
137. Hicke, L. & Dunn, R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* **19**, 141-72 (2003).
138. Motley, A., Bright, N. A., Seaman, M. N. & Robinson, M. S. Clathrin-mediated endocytosis in AP-2-depleted cells. *J Cell Biol* **162**, 909-18 (2003).
139. Hinrichsen, L., Harborth, J., Andrees, L., Weber, K. & Ungewickell, E. J. Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J Biol Chem* **278**, 45160-70 (2003).
140. Owen, D. J., Collins, B. M. & Evans, P. R. Adaptors for clathrin coats: structure and function. *Annu Rev Cell Dev Biol* **20**, 153-91 (2004).
141. Conner, S. D., Schroter, T. & Schmid, S. L. AAK1-mediated micro2 phosphorylation is stimulated by assembled clathrin. *Traffic* **4**, 885-90 (2003).
142. Jackson, A. P. et al. Clathrin promotes incorporation of cargo into coated pits by activation of the AP2 adaptor micro2 kinase. *J Cell Biol* **163**, 231-6 (2003).
143. Ricotta, D., Conner, S. D., Schmid, S. L., von Figura, K. & Honing, S. Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. *J Cell Biol* **156**, 791-5 (2002).
144. Greenberg, M., DeTulleo, L., Rapoport, I., Skowronski, J. & Kirchhausen, T. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. *Curr Biol* **8**, 1239-42 (1998).
145. Rapoport, I., Chen, Y. C., Cupers, P., Shoelson, S. E. & Kirchhausen, T. Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site. *Embo J* **17**, 2148-55 (1998).
146. Rodionov, D. G. & Bakke, O. Medium chains of adaptor complexes AP-1 and AP-2 recognize leucine-based sorting signals from the invariant chain. *J.Biol.Chem.* **273**, 6005-6008 (1998).
147. ter Haar, E., Harrison, S. C. & Kirchhausen, T. Peptide-in-groove interactions link target proteins to the beta-propeller of clathrin. *Proc Natl Acad Sci U S A* **97**, 1096-100 (2000).
148. Miele, A. E., Watson, P. J., Evans, P. R., Traub, L. M. & Owen, D. J. Two distinct interaction motifs in amphiphysin bind two independent sites on the clathrin terminal domain beta-propeller. *Nat Struct Mol Biol* **11**, 242-8 (2004).
149. Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C. & Wakeham, D. E. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol* **17**, 517-68 (2001).
150. Kooijman, E. E., Chupin, V., de Kruijff, B. & Burger, K. N. Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic* **4**, 162-74 (2003).
151. Farsad, K. et al. Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J Cell Biol* **155**, 193-200 (2001).

152. Legendre-Guillemin, V., Wasiak, S., Hussain, N. K., Angers, A. & McPherson, P. S. ENTH/ANTH proteins and clathrin-mediated membrane budding. *J Cell Sci* **117**, 9-18 (2004).
153. Zimmerberg, J. & McLaughlin, S. Membrane curvature: how BAR domains bend bilayers. *Curr Biol* **14**, R250-2 (2004).
154. Rappoport, J. Z., Simon, S. M. & Benmerah, A. Understanding living clathrin-coated pits. *Traffic* **5**, 327-37 (2004).
155. Bishop, N. E. Dynamics of endosomal sorting. *Int Rev Cytol* **232**, 1-57 (2003).
156. Seabra, M. C. & Wasmeier, C. Controlling the location and activation of Rab GTPases. *Curr Opin Cell Biol* **16**, 451-7 (2004).
157. De Matteis, M. A. & Godi, A. PI-loting membrane traffic. *Nat Cell Biol* **6**, 487-92 (2004).
158. Birkeland, H. C. & Stenmark, H. Protein targeting to endosomes and phagosomes via FYVE and PX domains. *Curr Top Microbiol Immunol* **282**, 89-115 (2004).
159. Christoforidis, S. et al. Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nat Cell Biol* **1**, 249-52 (1999).
160. Christoforidis, S., McBride, H. M., Burgoyne, R. D. & Zerial, M. The Rab5 effector EEA1 is a core component of endosome docking. *Nature* **397**, 621-5 (1999).
161. Weber, T. et al. SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759-72 (1998).
162. Bonifacino, J. S. & Lippincott-Schwartz, J. Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol* **4**, 409-14 (2003).
163. Komada, M. & Soriano, P. Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. *Genes Dev* **13**, 1475-85 (1999).
164. Raiborg, C., Bache, K. G., Mehlum, A., Stang, E. & Stenmark, H. Hrs recruits clathrin to early endosomes. *Embo J* **20**, 5008-21 (2001).
165. Raiborg, C. et al. Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat Cell Biol* **4**, 394-8 (2002).
166. Katzmann, D. J., Odorizzi, G. & Emr, S. D. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* **3**, 893-905 (2002).
167. Bénaroch, P. et al. How MHC class II molecules reach the endocytic pathway. *EMBO J.* **14**, 37-49 (1995).
168. Davidson, H. W. Direct transport of newly synthesized HLA-DR from the trans-Golgi network to major histocompatibility complex class II containing compartments (MIICS) demonstrated using a novel tyrosine-sulfated chimera. *J.Biol.Chem.* **274**, 27315-27322 (1999).
169. Roche, P. A., Teletski, C. L., Stang, E., Bakke, O. & Long, E. O. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc Natl Acad Sci U S A* **90**, 8581-5 (1993).
170. Hansen, H. J. et al. Internalization and catabolism of radiolabelled antibodies to the MHC class-II invariant chain by B-cell lymphomas. *Biochem J* **320 (Pt 1)**, 293-300 (1996).

171. Wang, K., Peterson, P. A. & Karlsson, L. Decreased endosomal delivery of major histocompatibility complex class II-invariant chain complexes in dynamin-deficient cells. *J Biol Chem* **272**, 17055-60 (1997).
172. Pieters, J., Bakke, O. & Dobberstein, B. The MHC class II associated invariant chain contains two endosomal targeting siganls within its cytoplasmic tail. *J.Cell Sci.* **106**, 831-846 (1993).
173. Bremnes, B., Madsen, T., Gedde-Dahl, M. & Bakke, O. An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalization. *J Cell Sci* **107 (Pt 7)**, 2021-32 (1994).
174. Kang, S., Liang, L., Parker, C. D. & Collawn, J. F. Structural requirements for major histocompatibility complex class II invariant chain endocytosis and lysosomal targeting. *J Biol Chem* **273**, 20644-52 (1998).
175. Reusch, U., Bernhard, O., Koszinowski, U. & Schu, P. AP-1A and AP-3A lysosomal sorting functions. *Traffic* **3**, 752-61 (2002).
176. McCormick, P. J., Martina, J. A. & Bonifacino, J. S. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proc Natl Acad Sci U S A* **102**, 7910-5 (2005).
177. Dugast, M., Toussaint, H., Dousset, C. & Benaroch, P. AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. *J Biol Chem* **280**, 19656-64 (2005).
178. Wang, K., Peterson, P. A. & Karlsson, L. Decreased endosomal delivery of major histocompatibility complex class II-invariant chain complexes in dynamin-deficient cells. *J.Biol.Chem.* **272**, 17055-17060 (1997).
179. Liu, S. H., Marks, M. S. & Brodsky, F. M. A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. *J Cell Biol* **140**, 1023-37 (1998).
180. Brachet, V., Péhau-Amaudet, G., Desaymard, C., Raposo, G. & Amigorena, S. Early endosomes are required for major histocompatiblity complex class II transport to peptide-loading compartments. *Mol.Biol.Cell* **10**, 2891-2904 (1999).
181. Sevilla, L. M., Richter, S. S. & Miller, J. Intracellular transport of MHC class II and associated invariant chain in antigen presenting cells from AP-3-deficient mocha mice. *Cell Immunol* **210**, 143-53 (2001).
182. Caplan, S., Dell'Angelica, E. C., Gahl, W. A. & Bonifacino, J. S. Trafficking of major histocompatibility complex class II molecules in human B-lymphoblasts deficient in the AP-3 adaptor complex. *Immunol Lett* **72**, 113-7 (2000).
183. Nakayama, K. & Wakatsuki, S. The structure and function of GGAs, the traffic controllers at the TGN sorting crossroads. *Cell Struct Funct* **28**, 431-42 (2003).
184. Nielsen, M. S. et al. The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. *Embo J* **20**, 2180-90 (2001).
185. Chen, H. J., Yuan, J. & Lobel, P. Systematic mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor cytoplasmic domain - An acidic cluster containing a key aspartate is important for function in lysosomal enzyme sorting. *J.Biol.Chem.* **272**, 7003-7012 (1997).

186. Bremnes, B., Madsen, T., Gedde-Dahl, M. & Bakke, O. An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalization. *J.Cell Sci.* **107**, 2021-2032 (1994).
187. Watts, C. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu Rev Immunol* **15**, 821-50 (1997).
188. Watts, C. Antigen processing in the endocytic compartment. *Curr Opin Immunol* **13**, 26-31 (2001).
189. Bryant, P. W., Lennon-Dumenil, A. M., Fiebiger, E., Lagaudriere-Gesbert, C. & Ploegh, H. L. Proteolysis and antigen presentation by MHC class II molecules. *Adv Immunol* **80**, 71-114 (2002).
190. Watts, C. et al. Creation versus destruction of T cell epitopes in the class II MHC pathway. *Ann N Y Acad Sci* **987**, 9-14 (2003).
191. Blum, J. S. & Cresswell, P. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc.Natl.Acad.Sci.USAUSA* **85**, 3975-3979 (1988).
192. Amigorena, S. et al. Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J.Exp.Med.* **181**, 1729-1741 (1995).
193. Manoury, B. et al. Asparagine endopeptidase can initiate the removal of the MHC class II invariant chain chaperone. *Immunity* **18**, 489-98 (2003).
194. Newcomb, J. R. & Cresswell, P. Structural analysis of proteolytic products of MHC class II-invariant chain complexes generated in vivo. *J.Immunol.* **151**, 4153-4163 (1993).
195. Riese, R. J. et al. Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity* **4**, 357-66 (1996).
196. Brachet, V., Raposo, G., Amigorena, S. & Mellman, I. Ii chain controls the transport of major histocompatibility complex class II molecules to and from lysosomes. *J Cell Biol* **137**, 51-65 (1997).
197. Driessen, C. et al. Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. *J Cell Biol* **147**, 775-90 (1999).
198. Nakagawa, T. et al. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* **280**, 450-3 (1998).
199. Tolosa, E. et al. Cathepsin V is involved in the degradation of invariant chain in human thymus and is overexpressed in myasthenia gravis. *J Clin Invest* **112**, 517-26 (2003).
200. Shi, G. P. et al. Role for cathepsin F in invariant chain processing and major histocompatibility complex class II peptide loading by macrophages. *J Exp Med* **191**, 1177-86 (2000).
201. Ogrinc, T., Dolenc, I., Ritonja, A. & Turk, V. Purification of the complex of cathepsin L and the MHC class II-associated invariant chain fragment from human kidney. *FEBS Lett.* **336**, 555-559 (1993).
202. Guncar, G., Pungercic, G., Klemencic, I., Turk, V. & Turk, D. Crystal structure of MHC class II-associated p41 Ii fragment bound to cathepsin L reveals the structural basis for differentiation between cathepsins L and S. *Embo J* **18**, 793-803 (1999).
203. Fiebiger, E. et al. Invariant chain controls the activity of extracellular cathepsin L. *J Exp Med* **196**, 1263-9 (2002).

204. Kobayashi, T. et al. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. *Mol Biol Cell* **11**, 1829-43 (2000).
205. Boes, M. et al. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature* **418**, 983-8 (2002).
206. Chow, A., Toomre, D., Garrett, W. & Mellman, I. Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. *Nature* **418**, 988-94 (2002).
207. Kleijmeer, M. et al. Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *J Cell Biol* **155**, 53-63 (2001).
208. Murk, J. L. et al. Endosomal compartmentalization in three dimensions: implications for membrane fusion. *Proc Natl Acad Sci U S A* **100**, 13332-7 (2003).
209. Turley, S. J. et al. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* **288**, 522-7 (2000).
210. Pond, L. & Watts, C. Characterization of transport of newly assembled, T cell-stimulatory MHC class II-peptide complexes from MHC class II compartments to the cell surface. *J Immunol* **159**, 543-53 (1997).
211. Barois, N., de Saint-Vis, B., Lebecque, S., Geuze, H. J. & Kleijmeer, M. J. MHC class II compartments in human dendritic cells undergo profound structural changes upon activation. *Traffic* **3**, 894-905 (2002).
212. Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W. & Geuze, H. J. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci* **113 Pt 19**, 3365-74 (2000).
213. Zitvogel, L. et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* **4**, 594-600 (1998).
214. Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M. & Mellman, I. Activation of lysosomal function during dendritic cell maturation. *Science* **299**, 1400-3 (2003).
215. Wilson, N. S., El-Sukkari, D. & Villadangos, J. A. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood* **103**, 2187-95 (2004).
216. Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782-7 (1997).
217. Simonsen, A. et al. Polarized transport of MHC class II molecules in Madin-Darby canine kidney cells is directed by a leucine-based signal in the cytoplasmic tail of the beta-chain. *Journal of Immunology* **163**, 2540-8 (1999).
218. Bouillon, M. et al. Lipid raft-dependent and -independent signaling through HLA-DR molecules. *J Biol Chem* **278**, 7099-107 (2003).
219. Anderson, H. A., Hiltbold, E. M. & Roche, P. A. Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat Immunol* **1**, 156-62 (2000).
220. Poloso, N. J., Muntasell, A. & Roche, P. A. MHC class II molecules traffic into lipid rafts during intracellular transport. *J Immunol* **173**, 4539-46 (2004).

221. Karacsonyi, C., Knorr, R., Fulbier, A. & Lindner, R. Association of major histocompatibility complex II with cholesterol- and sphingolipid-rich membranes precedes peptide loading. *J Biol Chem* **279**, 34818-26 (2004).
222. Edidin, M. The state of lipid rafts: from model membranes to cells. *Annu Rev Biophys Biomol Struct* **32**, 257-83 (2003).
223. Lagerholm, B. C., Weinreb, G. E., Jacobson, K. & Thompson, N. L. Detecting microdomains in intact cell membranes. *Annu Rev Phys Chem* **56**, 309-36 (2005).
224. Kenworthy, A. K. et al. Dynamics of putative raft-associated proteins at the cell surface. *J Cell Biol* **165**, 735-46 (2004).
225. Dietrich, C., Yang, B., Fujiwara, T., Kusumi, A. & Jacobson, K. Relationship of lipid rafts to transient confinement zones detected by single particle tracking. *Biophys J* **82**, 274-84 (2002).
226. Douglass, A. D. & Vale, R. D. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell* **121**, 937-50 (2005).
227. Bouillon, M. & Mourad, W. M. [Major histocompatibility complex (MHC) class II: are lipid rafts the missing link?]. *Med Sci (Paris)* **19**, 988-93 (2003).
228. Al-Daccak, R., Mooney, N. & Charron, D. MHC class II signaling in antigen-presenting cells. *Curr Opin Immunol* **16**, 108-13 (2004).
229. Kropshofer, H. et al. Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nat Immunol* **3**, 61-8 (2002).
230. Irvine, D. J., Purbhoo, M. A., Krogsgaard, M. & Davis, M. M. Direct observation of ligand recognition by T cells. *Nature* **419**, 845-9 (2002).
231. Henne, C., Schwenk, F., Koch, N. & Moller, P. Surface expression of the invariant chain (CD74) is independent of concomitant expression of major histocompatibility complex class II antigens. *Immunology* **84**, 177-82 (1995).
232. Moldenhauer, G., Henne, C., Karhausen, J. & Moller, P. Surface-expressed invariant chain (CD74) is required for internalization of human leucocyte antigen-DR molecules to early endosomal compartments. *Immunology* **96**, 473-84 (1999).
233. Triantafilou, K., Triantafilou, M., Wilson, K. M., Cherry, R. J. & Fernandez, N. Intracellular and cell surface heterotypic associations of human leukocyte antigen-DR and human invariant chain. *Hum Immunol* **60**, 1101-12 (1999).
234. Patterson, G. H. & Lippincott-Schwartz, J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* **297**, 1873-7 (2002).
235. Patterson, G. H. & Lippincott-Schwartz, J. Selective photolabeling of proteins using photoactivatable GFP. *Methods* **32**, 445-50 (2004).
236. Wubbolts, R. et al. Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *J Cell Biol* **135**, 611-22 (1996).
237. Lee, W., Haslinger, A., Karin, M. & Tjian, R. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* **325**, 368-72 (1987).
238. Chu, W. A., Moehlenkamp, J. D., Bittel, D., Andrews, G. K. & Johnson, J. A. Cadmium-mediated activation of the metal response element in human neuroblastoma cells lacking functional metal response element-binding transcription factor-1. *J Biol Chem* **274**, 5279-84 (1999).

239. Mayo, K. E., Warren, R. & Palmiter, R. D. The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. *Cell* **29**, 99-108 (1982).
240. Lichtlen, P. et al. Target gene search for the metal-responsive transcription factor MTF-1. *Nucleic Acids Res* **29**, 1514-23 (2001).
241. Germain, R. N. & Hendrix, L. R. MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* **353**, 134-139 (1991).
242. Simonsen, A., Momburg, F., Drexler, J., Hammerling, G. J. & Bakke, O. Intracellular distribution of the MHC class II molecules and the associated invariant chain (Ii) in different cell lines. *Int Immunol* **5**, 903-17 (1993).
243. Gregers, T. F., Nordeng, T. W., Birkeland, H. C., Sandlie, I. & Bakke, O. The cytoplasmic tail of invariant chain modulates antigen processing and presentation. *Eur J Immunol* **33**, 277-86 (2003).
244. Taub, M. & Saier, M. H., Jr. An established but differentiated kidney epithelial cell line (MDCK). *Methods Enzymol* **58**, 552-60 (1979).
245. Royer-Pokora, B., Peterson, W. D., Jr. & Haseltine, W. A. Biological and biochemical characterization of an SV40-transformed xeroderma pigmentosum cell line. *Exp Cell Res* **151**, 408-20 (1984).
246. Murnane, J. P. & Sabatier, L. Chromosome rearrangements resulting from telomere dysfunction and their role in cancer. *Bioessays* **26**, 1164-74 (2004).

Manuscript

Invariant chain and internalization of mature MHC class II from the cell surface

Ole J. B. Landsverk[1], Tone F. Gregers[2], Even Wahlseng[3], Cathrine Fladeby[1], Tommy W. Nordeng[1] and Oddmund Bakke[1,4]

[1]Department of Molecular Biosciences, University of Oslo, Norway, [2]Department of Biochemistry, The Norwegian Radium Hospital, Norway, [3]National Cancer Institute, NIH, Bethesda, USA, [4]Department of Biomedicine, University of Bergen, Norway.

Abstract. The major histocompatibility (MHC) class II molecules present peptides to the T cell receptor on CD4+ lymphocytes. The diversity of the epitopes presented depends critically on the processing of the ingested antigens, and the endocytic pathway constitutes a set of increasingly protease-rich/acidic compartments to facilitate the progressive digestion of antigens. Targeting of newly synthesized MHC class II molecules to more acidic endosomal compartments depends on association with the invariant chain (Ii) molecule. Ii is synthesized in excess of class II and is also present in a MHC class II-free form. It is therefore possible that Ii may have other functions in post-endoplasmatic reticulum compartments. In this study we demonstrate the interaction between free Ii and mature MHC class II molecules present at the plasma membrane. Using a photoactivatable green fluorescent protein (PAGFP) fused to MHC class II in combination with an inducible expression system for Ii we show that excessive amounts of Ii causes an increased endocytosis of mature class II, and the redistribution of these to late endosomes and lysosomes. This secondary association will thereby expand the repertoire of peptides encountered by mature MHC class II molecules.

Introduction:

The MHC class II molecules are expressed as heterodimers of two transmembrane polypeptides. The α (35kD) and β (27kD) chains spontaneously associate in the endoplasmatic reticulum (ER)[1] via interactions between their transmembrane and lumenal domains. Correctly assembled heterodimers may traffic through the ER and Golgi apparatus to the plasma membrane. At the cell surface they may enter early endosomal compartments and load peptides for presentation to $CD4^+$ T lymphocytes. Correct assembly and transport of MHC class II is, however, greatly enhanced in the presence of Ii (Cresswell, 1994). Ii is a type II transmembrane glycoprotein that exists in different isoforms due to alternative splicing and use of alternative translation initiation start sites (Strubin et al., 1986; O'Sullivan et al., 1987). In the ER, Ii forms trimers which associate with three class II heterodimers forming a nonameric class II-Ii complex $(\alpha\beta Ii)_3$.

Address all correspondence to Oddmund Bakke, Department of Molecular Biosciences, University of Oslo. Tel.: +47 22855787. Fax: +47 22854605. E-mail: oddmund.bakke@imbv.uio.no

1. Abbreviations used in this paper: APC, antigen presenting cell; CLIP, class II associated invariant chain peptide; DC, dendritic cell; ER, endoplasmatic reticulum; ILEV, Ii induced large endosomal vesicles; Ii, invariant chain

The association is driven by several class II–Ii interacting domains, with the most prominent being the class II interacting invariant chain peptide (CLIP) (Freisewinkel et al., 1993; Romagnoli and Germain, 1994). CLIP binds in the peptide binding grove of class II, stabilizing the heterodimer (Anderson and Miller, 1992), and preventing premature peptide binding (Roche and Cresswell, 1990; Busch et al., 1996).

Two leucine-based sorting signals in the Ii cytoplasmic tail (Pieters et al., 1993; Bremnes et al., 1994; Odorizzi et al., 1994) target the $(\alpha\beta Ii)_3$ complex to the endosomal/lysosomal system where Ii is sequentially degraded from the lumenal side (Watts, 2001), leaving only CLIP bound to the peptide binding grove of the αβ heterodimer. Exchange of CLIP for antigenic peptide is facilitated by the catalytic action of HLA-DM, which acts as a peptide editor, promoting the loading of tightly binding peptides (Brocke et al., 2002). Peptide loading onto MHC class II molecules has been found to take place throughout the endocytic pathway, although several characteristics have been defined for the compartments where peptide loading can occur (Neefjes, 1999).

The MHC class II heterodimer also contains signals for endosomal sorting (Salamero et al., 1990; Simonsen et al., 1993; Humbert et al., 1993) and can be internalized and recycled in the absence of Ii. Pinet *et al*. (Pinet et al., 1995) have shown that the cytoplasmic tails of the αβ heterodimers are essential for this routing, Zhong *et al*. (Zhong et al., 1997) reported that the LL sequence in the C-terminus of the murine β chain is a sorting signal, and the HLA-DRβ chain was confirmed to contain a corresponding signal (Simonsen et al., 1999). Cells expressing class II molecules in the absence of Ii can present certain exogenous peptides efficiently (Miller and Germain, 1986; Sekaly et al., 1986; Anderson et al., 1993; Nijenhuis et al., 1994; Pinet et al., 1995), demonstrating that recycling class II molecules can reach endosomes containing degraded antigens. Recycling class II molecules, however, preferentially bind peptides in early endosomes (Pinet and Long, 1998), whilst newly synthesized MHC class II molecules bind peptides in later compartments (Zhong et al., 1997). The endocytic pathway exposes internalized antigens to a progressively lower pH and to higher concentrations of proteases (Trombetta and Mellman, 2005). Newly synthesized and recycled MHC class II molecules would therefore be expected to present different peptides (Pinet et al., 1995; Zhong et al., 1997). It is, however, still unclear whether mature class II molecules that recycle at the plasma membrane may reach more acidic compartments, or if they are restricted to early 'recycling' endosomes. The class II associated invariant chain is in antigen presenting cells (APCs) produced in excess of MHC class II α and β chains (Machamer and Cresswell, 1982; Kvist et al., 1982; Nguyen and Humphreys, 1989; Kampgen et al., 1991). It has previously been shown that the free pool of Ii can modulate antigen processing and presentation by reducing degradation of antigen and Ii itself (Gorvel et al., 1995; Gregers et al., 2003). MHC class II associated- and free Ii follows the same pathway to late endosomes/lysosomes, mainly (Roche et al., 1993; Hansen et al., 1996; Wang et al., 1997), if not exclusively (Dugast et al., 2005; McCormick et al., 2005), via the plasma membrane. At the cell surface they are rapidly internalized, with a half-life of 1-5 minutes (Bakke and Dobberstein, 1990; Roche et al., 1993; Bremnes et al., 1994), due to the strong internalization signal in the cytoplasmic tail of Ii (Kang et al., 1998). A secondary 'post-ER' interaction between free-Ii and mature class II could provide the mechanism for sorting mature class II deeper into the endocytic pathway. The intimate relationship of Ii with newly synthesized class II has however, made it difficult to separate the primary interaction in the ER and a potential secondary post-ER interaction. In this study we have taken advantage of the PAGFP (Patterson and Lippincott-Schwartz, 2002), and fusion of this to MHC class II has enabled us to show how an inducible invariant chain caused the internalization of mature class II to late endosomes and lysosomes.

Results:

In order to examine whether the free pool of Ii can affect the trafficking of mature MHC class II molecules, we utilized MDCK and/or M1 cells constitutively expressing mouse or human MHC class II, I-Ak and HLA-DR respectively, and Ii p33 under control of a metallothionein promoter. I-Ak has previously been shown to associate with human Ii (Simonsen et al., 1993) and Ii p33 is the major Ii isoform expressed in APCs (Strubin et al., 1986; O'Sullivan et al., 1987). Most of the results presented in this study were done in parallel using both cell types and both human and mouse MHC class II, where otherwise the cell- and class II-type is indicated. Ii constructs used in this study are listed in Figure 1.

To initially establish the effect of saturating amounts of Ii, we performed an over-night incubation with CdCl2. We found that increasing expression levels of Ii redistributes MHC class II from the plasma membrane to intracellular compartments. To assess the relative time-frame of this redistribution, we fixed the cells at different time points after Ii induction. This revealed that after 4 hours, there was a marked decrease of MHC class II at the cell surface and after 6 hours it was mainly localized to intracellular compartments (unpublished data, but Figure 3A contains similar results). The sorting signals in the cytoplasmic tail of Ii have been shown to affect various transport steps from the ER to the endocytic pathway. To confirm that the depletion of class II from the plasma membrane was dependent on the endosomal sorting function of Ii, we performed the same experiment with an Ii construct lacking the

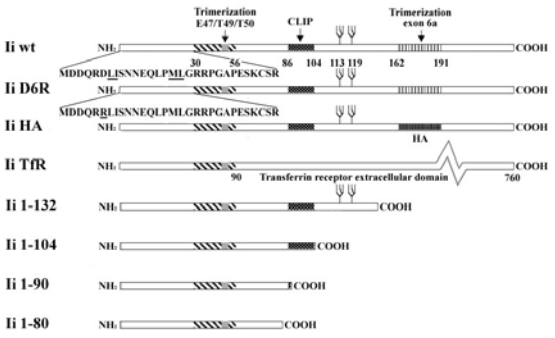

Figure 1 Ii constructs used in the study. Ii D6R was created by site-directed mutagenesis on the cytoplasmic tail of Ii. Ii HA has a sequence derived from hemagglutinine in place of CLIP and was generated by PCR-soeing. The TfR-Ii fusion protein consists of the cytoplasmic tail and transmembrane domains from human Ii (p33) and the lumenal domain of the transferrin receptor. The C-terminal deletion mutants were generated by PCR (using pSV51L-Ii as a template). All constructs were subcloned into pMep4 as KpnI-BamHI fragments.

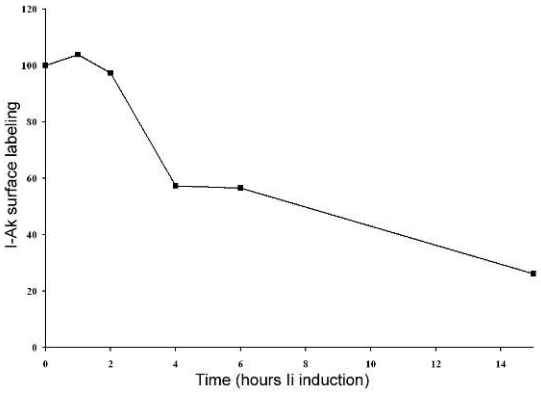

Figure 2 Quantification of Ii mediated class II redistribution. $MDCK_{I\text{-}Ak/Ii\text{-}wt}$ cells were incubated with 25μM $CdCl_2$ for 0, 1, 3, 6 and 15 hours before they were placed on ice, washed in ice cold PBS and labeled with 2μg/ml ^{125}I-H116-32 for 45minutes. The cells were then washed extensively with PBS/BSA prior to acetic acid treatment to collect the surface bound ^{125}I-H116-32. The acid wash was collected and counted in a COBRA γ

cytoplasmic tail ($Ii_{\Delta 20}$)(Bakke and Dobberstein, 1990). MHC class II remained at the plasma membrane, even after 6 hours, where it colocalized with $Ii_{\Delta 20}$, showing that the internalization was dependent on intact sorting signals in Ii (unpublished data).

To quantify the observed Ii mediated redistribution of MHC class II we incubated $MDCK_{I\text{-}Ak/Ii\text{-}wt}$ cells with iodinated anti-I-Ak antibodies at different time-points after Ii induction (Figure 2). We found that after 3 hours of induction, I-Ak was still predominately expressed at the plasma membrane whilst after 4 hours surface labeling had decreased by 45%. After 15 hours of Ii induction, only 25% of total MHC class II was found at the cell surface. These results show that between 3 and 4 hours Ii causes a significant reduction of class II at the plasma membrane. However, newly synthesized MHC class II would be expected to contribute to the total amount of cell surface class II and to investigate this contribution we attached PAGFP to the c-terminus of the HLA-DR α-chain and generated MDCK cells stably expressing $HLA\text{-}DR_{PAGFP}$ and inducible Ii. By photoactivating class II and inducing Ii after 1 hour (to allow egress of the now fluorescent MHC class II from the ER), we were able to exclude imaging newly synthesized, Ii associated, class II. As shown in figure 3A, the photoactivated MHC class II are redistributed from the plasma membrane to intracellular compartments, were they colocalize with Ii, eventually forming the characteristic Ii induced large endosomal vesicles (ILEVs). The time frame of the "take-down" was seen to increase dramatically after 3-4 hours, thus supporting our previous result. After 12 hours hardly any photoactivated class II was present at the cell

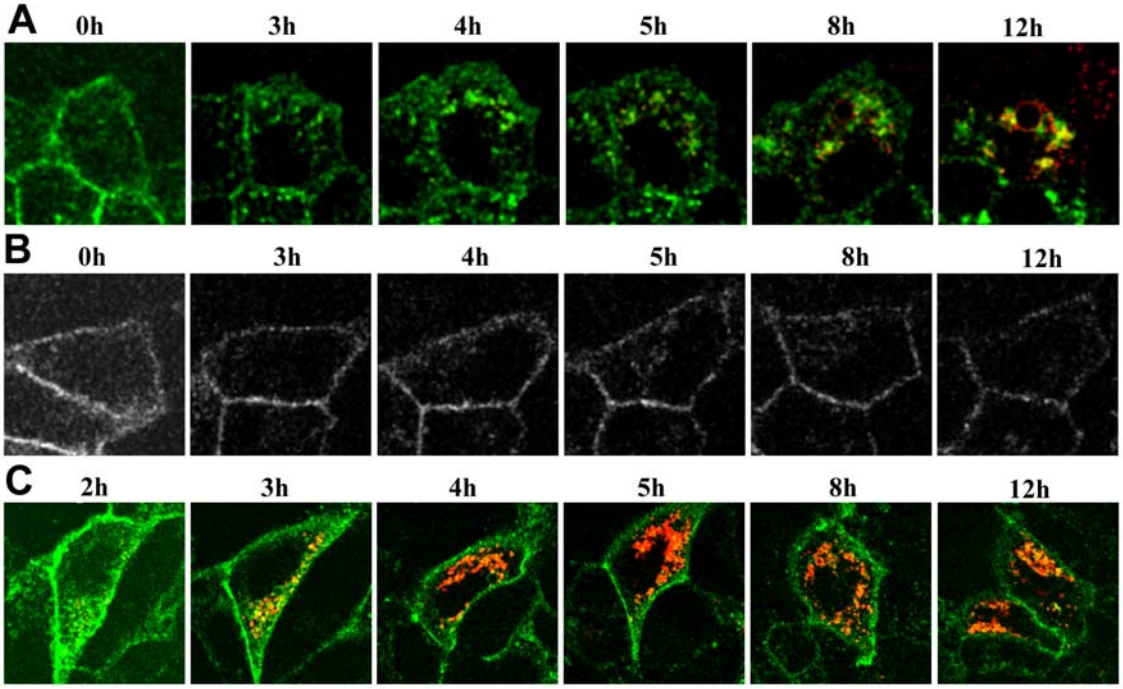

Figure 3 Distribution of photoactivated MHC class II with induction of Ii (A), without Ii (B) and with indused TfR-Ii (C). $MDCK_{Ii+\beta\alpha PA}$ (A) and $MDCK_{\beta\alpha PA}$ (B) were incubated with BU45 for 30 minutes and MHC class II was photoactivated with the 405nm laser and allowed to exit the ER for 1 hour before 15μM $CdCl_2$ was added to the medium to induce Ii expression (A) or observe unspecific bi-effects of Cd^{2+} (B). $MDCK_{\beta\alpha PA}$ were transiently transfected with TfR-Ii, and transferrin-alexa647 and 15μM $CdCl_2$ was added to the medium 2 hours before imaging to detect TfR-Ii positive cells (C). MHC class II was photoactivated and imaged as above. Images obtained every 3 minutes, images from selected time points shown.

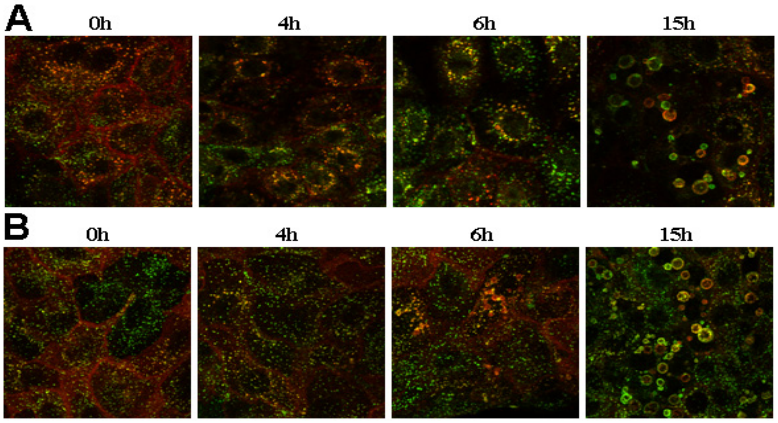

Figure 4 A) Ii transports MHC class II to late endosomes and lysosomes, B) The redistribution to LAMP1 positive compartments is independent of the sorting signals in MHC class II. $MDCK_{I\text{-}Ak/Ii\text{-}wt}$ (A) or $MDCK_{HLA\text{-}DR\Delta/Ii\text{-}wt}$ (B) were incubated with 25μM $CdCl_2$ for 0, 4, 6 and 15 hours and then fixed in 3% PFA. The cells were then labeled with the anti I-Ak antibody H116-32 (A) or the anti HLA-DR antibody L243 (B) and anti LAMP1 before labeling with secondary antibodies goat anti-mouse IgG Alexa594 (MHC class II) and goat anti-rabbit IgG Alexa488 (LAMP1). Cells containing ILEVs are expressing Ii.

surface.

In cells not expressing Ii, but otherwise treated in the same manner, the labeled class II molecules remained at the cell surface for the duration of the experiment (Figure 3B). This shows that it is indeed mature MHC class II that under the influence of Ii is internalized to endosomes.

Ii has fusogenic properties, when over expressed in cells it causes the generation of enlarged endosomal vesicles (ILEVs) (Stang and Bakke, 1997). Thus, it could be speculated that these might capture recycling MHC class II molecules. To examine the contribution of such an effect, we transfected cells with an Ii mutant (IiD6R) that is unable to cause endosomal retention (Nordeng et al., 2002). We found that the class II redistribution from the plasma membrane to endosomes was the same as for Ii_{wt}, indicating that the membrane capturing properties of Ii do not contribute to the effect.

Ii contains within its cytoplasmic tail two strong internalization motifs, both independently sufficient for endocytosis at the plasma membrane. It could therefore be speculated that the internalization was caused by the distribution of MHC class II to membrane domains involved in increased endocytosis. To investigate any such contribution, we transfected the $HLA\text{-}DR_{PAGFP}$ cells with a chimeric protein containing the extracellular domain of the transferrin receptor (TfR) and the intact transmembrane and cytosolic domains of Ii (TfR-Ii) (Odorizzi et al., 1994). As shown in Figure 3C, the chimera was unable to deplete class II from the plasma membrane. This indicates that also the lumenal domain of Ii is required for the redistribution, and that the internalization is dependent on a specific interaction between Ii and mature class II.

Newly synthesized MHC class II molecules are transported to late endosomes and lysosomes by Ii.

To examine whether the internalized class II molecules are targeted to the same compartments, we incubated cells with $CdCl_2$ for different time intervals, fixed the cells and stained with anti-LAMP1 antibodies (Figure 4A). In the absence of Ii, class II was mainly localized to the plasma membrane, with a very minor fraction in LAMP1 positive compartments. After 4 and 6 hours, MHC class II was depleted from the cell surface and the majority of MHC class II could be seen in LAMP1 positive compartments. After 15 hours of Ii induction MHC class II was found almost exclusively in ILEVs where it colocalized with LAMP1.

The leucine based signal in the β chain mediates internalization and recycling at the cell surface and to assess the contribution of this signal we expressed Ii in cells with tailless HLA-DR molecules ($HLA\text{-}DR_{\Delta}$). We found that $HLA\text{-}DR_{\Delta}$ localized to the cell surface in the absence of Ii, but after Ii induction $HLA\text{-}DR_{\Delta}$ was relocated to LAMP1 positive compartments with similar kinetics as $HLA\text{-}DR_{wt}$ (Figure 4B). This shows that the internalization and trafficking to late endosomes/lysosomes is independent of any contribution by the sorting signal in the MHC class II cytoplasmic tails.

Ii associates with the class II molecules via several interacting domains, the most important being the CLIP region. The peptide binding groove of the MHC class II present at the cell surface would however, for the majority of the class II molecules at least (Vacchino and McConnell, 2001), be occupied by peptides as empty class II are unstable (Rabinowitz et al., 1998), and prone to be sorted to lysosomes and degraded. It has been shown that the transmembrane domain of Ii contributes towards class II association (Castellino et al., 2001). However, the TfR-Ii chimera demonstrated that this domain was insufficient for a secondary 'post-ER' interaction and consequent plasma membrane clearance of MHC class II. To determine which regions of Ii N-terminal to CLIP are required to achieve MHC class II redistribution, we generated two Ii deletion mutants where the C-terminal regions were removed, $Ii_{1\text{-}90}$ and $Ii_{1\text{-}80}$ (Figure 1). The constructs were transfected into cells, but both $Ii_{1\text{-}90}$ and $Ii_{1\text{-}80}$ were retained in the ER, as observed by immunofluorescence microscopy

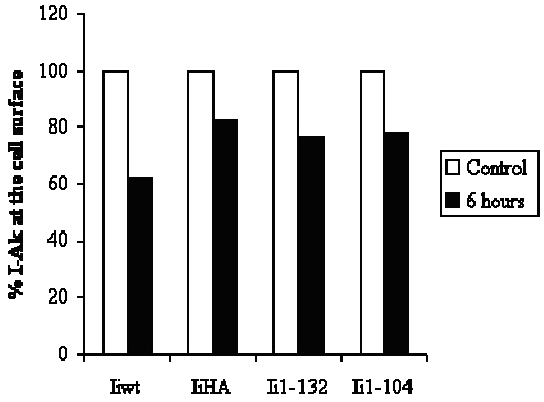

Figure 5. Quantification of Ii-mutant mediated class II redistribution. A) Ii C-terminal deletion mutants were created by PCR, subcloned into pMEP4 and transfected into MDCK I-Ak cells. B) Cells expressing Iiwt, IiHA, Ii1-104 and Ii1-132 were incubated with 25µM CdCl2 for 0 and 6 hours and then washed and incubated on ice with 125I-H116-32 for 45 minutes. The cells were then washed extensively with PBS/BSA prior to acetic acid treatment to collect the surface bound 125I-H116-32. The acid wash was collected and counted in a COBRA γ counter. The results are given as mean duplicates were the value received at T=0 is set to 100%

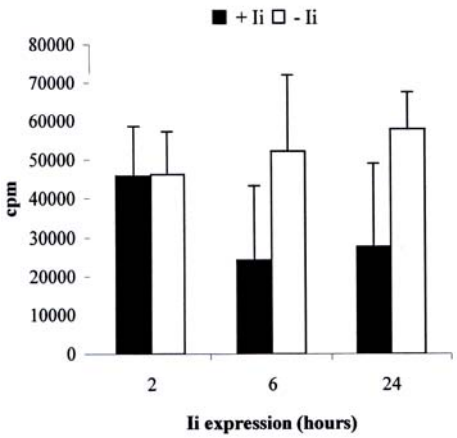

Figure 6 Ii mediated depletion of MHC class II results in less efficient activation of naïve T cells. MDCKI-Ak/Ii-wt cells were incubated with 25µM CdCl2 for 0, 6 and 24 hours to induce Ii expression before they were fixed in 1% PFA and incubated with 10µg/ml synthetic HEL 116-129 peptides and 5x104 1B9 T cell hybridomas over night. IL-2 production from the 1B9 cells was measured indirectly by proliferation of the IL-2 dependent cell line CTLL-2. The results are obtained from one experiment done in triplicates.

(unpublished data). We therefore decided to study the effect of CLIP and the other C-terminal parts of Ii separately, and to this effect we used a Ii construct in which CLIP was replaced with an irrelevant sequence derived from hemagglutinine (HA; YPYDVPDYA). This peptide is unlikely to associate with the peptide binding groove of I-Ak as it lacks the major anchor residues required for high affinity binding (Fremont et al., 1998). However, Ii_{HA} was still able to deplete I-Ak from the plasma membrane as observed by immunofluorescence microscopy (unpublished data). To obtain quantitative data on the effect of Ii_{HA}, MDCK cells were induced to express Ii_{HA} for 6 hours prior to incubation with iodinated anti I-Ak antibodies on ice. Ii_{HA} was somewhat less efficient than Ii_{wt}, but still able to cause internalization, indicating that association via the CLIP region is not a prerequisite for cell surface clearance of MHC class II (Figure 5).

Thayer *et al*. (Thayer et al., 1999) have shown that a region C-terminal to CLIP might interact outside of the peptide binding groove under conditions where CLIP has little or no affinity for the groove. To examine whether this region contributes to the internalization of mature class II we made two new deletion mutants, Ii_{1-104} and Ii_{1-132} (Figure 1), which we transfected into cells. Both mutants colocalized with class II in endosomes, indicating that they exhibited normal trafficking, and both were able to redistribute class II within 6 hours, as observed by immunofluorescence microscopy, although Ii_{1-104} appeared less efficient compared to Ii_{wt} and Ii_{1-132} (unpublished data). Quantification of surface I-Ak before and after induction however, revealed that both caused increased internalization of I-Ak, although somewhat less than Ii_{wt} (Figure 5). Thus neither CLIP nor amino acids 105-216 are an absolute requirement for internalization of MHC class II. $CD4^+$ T cells recognize antigenic peptides in the context of MHC class II and to further confirm that the amount of class II at the plasma membrane is reduced upon Ii expression, $MDCK_{I\text{-}AK/Ii\text{-}wt}$ cells were induced to express Ii for 2, 6 and 24 hours and consequently fixed in PFA. The cells were then incubated with synthetic HEL 116-129 peptide and the 1B9 T cell hybridoma. IL-2 production from 1B9 was measured indirectly by proliferation of the IL-2 dependant CTLL-2 cell line. As shown in figure 6, absence of Ii gave efficient loading and presentation of the HEL 116-129 epitope, whilst after 6 and 24 hours of Ii expression activation of 1B9 T cells was reduced. This then supports our previous results, showing that Ii relocates MHC class II from the cell surface to intracellular compartments resulting in reduced amounts of surface class II and less efficient presentation of the HEL 116-129 peptide to HEL specific T cells.

Discussion:

There is at present a general consensus that at least the vast majority of newly synthesized $(\alpha\beta Ii)_3$ complexes are transported via the plasma membrane to compartments where peptide loading can occur (Roche et al., 1993; Hansen et al., 1996; Wang et al., 1997; Dugast et al., 2005; McCormick et al., 2005). At the cell surface both free Ii and $(\alpha\beta Ii)_3$ complexes

are rapidly internalized, whereas MHC class II unassociated with Ii may reside stably at the plasma membrane for several days (Cella et al., 1997). MHC class II and Ii are under subtly different transcriptional regulation (Peijnenburg et al., 1999) and there are reports which may indicate that also their mRNA stability may be variably controlled(Shih et al., 1997; Kuchtey et al., 2003). The class II transactivator (CIITA) can by itself cause the upregulation of both Ii and class II (Steimle et al., 1994). The general higher expression level of Ii would ensure that enough Ii is available for efficient assembly and transport of newly synthesized class II in the ER. However as Ii is transported to the plasma membrane and the endosomal pathway in the absence of class II, it is possible that it may be further involved in modulating MHC class II transport and distribution. Mature class II molecules at the cell surface may enter endosomal recycling compartments. These MHC class II molecules may exchange/load peptides, but are not exposed to the wide repertoire that newly synthesized, Ii associated, class II molecules encounter in later endosomal compartments.

Henne (Henne et al., 1995) and later Moldenhauer (Moldenhauer et al., 1999) and Triantafilou (Triantafilou et al., 1999) speculated that free Ii could associate with empty αβ dimers and promote their endocytosis and delivery to processing compartments. However, as they were not convincingly differentiating between an association in the ER and at the plasma membrane in their studies, this model has not been implicated in MHC class II trafficking. In our system we are able to discriminate definitively between the two. By using a PAGFP-tagged MHC class II, and an inducible expression system for Ii, we are able to exclude imaging the newly synthesized $(\alpha\beta Ii)_3$ complexes. This allows us to specifically image mature class II and the effect of increasing amounts of Ii on them.

Data presented in this study show that free-Ii appearing at the plasma membrane can cause the endocytosis and transport of mature MHC class II to more acidic/protease rich compartments. Ii will therefore expand the array of peptides encountered by mature class II molecules. The redistribution is dependent on the intact cytoplasmic tail of Ii, and rapid, with a dramatic increase in the internalization rate 3 to 4 hours after Ii induction. This time most probably correlates to the time that Ii saturates available class II in the ER and appears in large numbers at the PM alone. Ii is itself rapidly internalized at the PM, and would be presumed to capture and internalize class II with similar kinetics.

Elevated expression of Ii has been shown to induce endosomal retention (Romagnoli et al., 1993; Pieters et al., 1993; Gorvel et al., 1995; Stang and Bakke, 1997), and the clearance of class II from the cell surface could be thought to be due to retention in the recycling compartment. This would prevent return of recycling MHC class II molecules to the cell surface. However, under these conditions we have previously shown that the recycling pathway is unaffected, at least for the TfR (Gregers et al., 2003). In addition, an Ii mutant (IiD6R) that is unable to cause endosomal retention (Nordeng et al., 2002) was still able to cause the redistribution of MHC class II. Ii harbors in its N-terminal cytoplasmic tail two signals that mediate rapid endocytosis at the plasma membrane (Pieters et al., 1993; Bremnes et al., 1994; Odorizzi et al., 1994), and the decrease in class II at the cell surface could possibly be caused by a unspecific internalization caused by increased endocytosis. However a TfR-Ii chimera (Odorizzi et al., 1994) with Iis cytoplasmic tail does not deplete class II from the PM.

The major class II-Ii interaction site is the CLIP region. However, for the majority of class II molecules present at the cell surface the peptide-binding groove should be occupied (Vacchino and McConnell, 2001). The interaction was therefore presumed to involve different domains of Ii, and to examine this several C-terminal Ii deletion mutants were made and analyzed by both immunofluorescence microscopy and quantitatively by ^{125}I labeling. Both Ii_{1-104} and Ii_{1-132} were able to cause the internalization of MHC class II, but somewhat less efficiently than Ii_{wt}. Nevertheless we could conclude that none of the residues contained within amino acids 105-216 are absolutely required for the internalization. An additional Ii mutant with a sequence derived from hemagglutinine replacing CLIP was also able to redistribute class II, although significantly less than Ii_{wt}, suggesting that the CLIP region may contribute to the interaction. As it is unlikely that this interaction is via the peptide-binding groove of surface MHC class II, we speculate that CLIP might impose a particular conformation onto neighboring regions which promotes their interaction with the class II molecule. All in all we did not find that one specific domain of Ii was responsible for the internalization, but rather that the various class II interacting domains of Ii contribute and sum up to the effect. Considering the diversity of MHC class II alleles, a number of interaction sites would better facilitate the general mechanism, ensuring that the wide array of MHC class II alleles would be susceptible to this secondary interaction with Ii.

The mechanism we describe could serve at least 3 possible functions. The most obvious is to ensure the presence of class II molecules throughout the endocytic pathway upon the initiation of an immune response. However, it is also tempting to speculate whether this ability of Ii might be a factor involved in clearance of self-peptide loaded MHC class II after initiation of an immune response. The transcription of class II genes has been shown to be transiently upregulated after activation of dendritic cells (DCs), followed by a complete shut down (Cella et al., 1997;

Rescigno et al., 1998; Landmann et al., 2001; Pai et al., 2002; Wilson et al., 2004). Considering the in general elevated expression of Ii as compared to MHC class II, this could provide an opportunity for replacement of these potentially autoimmune provoking molecules. Certainly this would not be the only mechanism operating, considering the general redundancy in biological systems, and the potential importance of such a mechanism in relation to autoimmunity. Indeed, DCs isolated from Ii null mice have been shown to display errant transport of some MHC class II alleles whilst others traffic normally and display MHC class II as $Ii^{+/+}$cells (Rovere et al., 1998). The numerous studies implying variations in endosomal pH, alterations in cathepsin activity (Trombetta et al., 2003) and Ii processing (Pierre and Mellman, 1998) following activation of DCs provide additional incentive for considering this property of Ii in relation to this proposed mechanism.

Adding to this, it can not be excluded that Ii might contribute to the retention of MHC class II intracellularly in immature DCs (reviewed in references) (Trombetta and Mellman, 2005; Wilson and Villadangos, 2005). Certainly a high expression level of Ii, as has been described for imDCs (Kampgen et al., 1991), would ensure a rapid turnover of cell surface class II. A transient upregulation upon activation would facilitate removal of self-peptide loaded class II. And a shut-down of class II gene transcription would, after Ii degradation, enable the significantly longer-lived class II molecules to transport to, and remain stably at, the cell surface. At the cell surface they would then present the widest possible array of peptides, as promoted by Ii directed trafficking.

In summary, we show that Ii in excess is able to accelerate the endocytosis of mature, cell surface expressed, MHC class II. The effect is specific for class II, and not a bi-effect of Iis membrane acquiring properties. The internalized class II molecules are distributed to intracellular compartments where they colocalize with the lysosomal marker LAMP1. We find that the internalization is independent on sorting signals in MHC class II, but dependant on intact signals in Ii. And we find that the various class II interacting lumenal domains of Ii contribute and sum up to the effect.

Materials and methods:

cDNA constructs:

The plasmids pFM339 and pFM340, containing I-Akα and I-Akβ respectively, are derived from the eukaryotic expression vector pHbetaAPr-1-neo and have been previously described(Gregers et al., 2003). HLA-DR1α and HLA-DR3β were obtained from the HLA-DR1a and # 69 DRα IRES DR3β-CFP (kindly provided by Alexander Griekspoor) constructs respectively. HLA-DR1α was subcloned into pPAGFP-N1 (courtesy of Jennifer Lippincott-Schwartz) using EcoRI-BamHI restriction sites, generating a fusion protein with a DPPVAT linker sequence. HLA-DR3 β was subcloned into pcDNA3 (Invitrogen) as a HindIII-EcoRI fragment. The TfR-Ii fusion protein consists of the cytoplasmic tail and transmembrane domains from human Ii (p33), fused in-frame to the luminal part of the human transferrin receptor, and subcloned into pMEP4 as a KpnI-BamHI fragment. cDNA encoding Ii-wt, IiD6R and IiΔ20 have been described previously (Bakke and Dobberstein, 1990; Simonsen et al., 1997; Gregers et al., 2003), and were subcloned into the pMep4 vector (Invitrogen) as KpnI-BamHI fragments. pMep4 with an Ii construct where the CLIP region is replaced with a sequence derived from hemagglutinine (HA); YPYDVPDYA, was a gift from Vibeke Årskog (University of Oslo, Norway). The C-terminal deletion mutants were generated by PCR (using pSV51L-Ii as a template) and subcloned into pMep4 as KpnI-BamHI fragments. All constructs were verified by sequencing (GATC Biotech, Konstanz, Deutschland).

Cell lines:

Madin-Darby Canine Kidney (MDCK) strain II cells and human fibroblast (M1) cells were grown in complete medium: DMEM (Bio Whittaker, MD, USA) supplemented with 10% FCS (Integro b.v., Zaandam, Netherlands), 2 mM L-glutamine, 25 U/ml penicillin and 25 µg/mL streptomycin (Bio Whittaker). MDCK cells stably transfected with human or mouse MHC class II, with or without Ii, $MDCK_{HLA\text{-}DR/Ii\text{-}wt}$, $MDCK_{HLA\text{-}DR\Delta/Ii\text{-}wt}$ and $MDCK_{I\text{-}Ak/Ii\text{-}wt}$, $MDCK_{I\text{-}Ak}$, respectively, have been described previously (Simonsen et al., 1999; Gregers et al., 2003). These cells were grown in complete medium containing 0.2 mg/mL G418 bisulphate (Duchefa, Haarlem, Netherlands) and 0.15 mg/mL Hygromycin B (Saaven, Sweden). Stable transfections were carried out with the Lipofectamin 2000 reagent (Invitrogen) according to supplied protocol. Positive clones were selected in 0.3 mg/mL G418, 0.3 mg/mL Hygromycin B, expression of ILEVs after adding 25 µM CdCl2 over-night, or fluorescent signal. Clones expressing the relevant constructs were picked with cloning cylinders, generating the $MDCK_{\beta\alpha PA}$, $MDCK_{Ii+\beta\alpha PA}$, $M1_{HLA\text{-}DR}$, $M1_{HLADR\Delta}$ and $M1_{I\text{-}Ak}$, cell-lines.

Antibodies, antigens, fluorescent probes and T cells:

BU43 is a mouse IgM monoclonal antibody (mAb) specific for the C-terminal (lumenal/extracellular) domain of Ii (D. Harding, Birmingham, UK). The BU45-HPSF1 hybridoma was kindly supplied by S. Buus (Copenhagen, Denmark), it produces the mouse mAb BU45 (Wraight et al., 1990) which also recognizes the C-terminal domain of Ii. BU45 was directly labeled with alexa 546 (Molecular Probes, Eugene, UK) according to the supplied protocol. The rabbit anti-LAMP1 polyclonal antibody was a gift from S. Carlsson (Umeå, Sweden). H116-32 is a mouse monoclonal IgG2b antibody specific for mouse I-Ak (Koch et al., 1982), and was provided by F. Momburg (Heidelberg, Germany). Mouse monoclonal L243 (American Type Culture Collection, Manassas, VA, USA) recognizes HLA-DR (Lampson and Levy, 1980). Goat anti-mouse IgG Alexa 488/594, goat anti-rabbit Alexa 488 and Transferrin-alexa 647 conjugate were purchased from Molecular probes (OR, USA). Texas-red conjugated goat anti-mouse IgM was obtained from Southern Biotechnology Associates Inc. (AL, USA). IB9 is a T cell hybridoma which recognizes a peptide derived from Hen Egg Lysozyme (HEL, 116-129) bound to I-Ak (Zhong et al., 1997). 1B9 T cell hybridoma and synthetic peptide were both provided by R. Germain (Bethesda, MD, USA).

Immunofluorescence- and confocal laser scanning microscopy:

Cells were grown confluent on coverslips and induced to express Ii with 25µM $CdCl_2$ for the defined time periods, then allowed to internalize BU43 prior to fixation in 3% paraformaldehyd (PFA). Cells were stained with the appropriate primary and secondary antibodies in 0.1% saponine in 1xPBS before mounting in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL, USA) or Moviol medium (Sigma-Aldrich). Immunofluorescence microscopy was performed on a Leica TCS-NT digital scanning confocal microscope equipped with a 60/1.2

immersion objective (Leica, Bensheim, Germany) or an Olympus FV1000 scanning laser confocal microscope equipped with a 60/1.1 oil immersion objective (Olympus, Hamburg, Germany). Fluorochromes were exited with the appropriate laser lines; 488 nm Argon, 543 nm and 647 nm HeNe lasers. PAGFP was activated with the 405 nm laser and imaged as GFP with the 488 nm argon laser. For the "Ii chase" time-lapse imaging experiments, $MDCK_{Ii+\beta\alpha PA}$ and $MDCK_{\beta\alpha PA}$ were seeded out in glass bottom microwell dishes (MatTek Corporation, Ashland, USA), medium was exchanged for DMEM without phenol red (Bio Whittaker, MD, USA) supplemented with FCS, LG and PS and added the BU45-alexa546 conjugate 1 hour before imaging. MHC class II-PAGFP was photoactivated and allowed to exit the ER/distribute to the cell surface for 1 hour before Ii was induced. The TfR-Ii chase was conducted on the $MDCK_{\beta\alpha PA}$ cells transiently transfected with the TfR-Ii construct, and 15μM $CdCl_2$ and the transferrin-alexa 647 conjugate was added to the medium for 2 hours before imaging to detect positive cells. All time-lapse imaging was conducted on the Olympus (se above) with images obtained every 3 minutes at 1-1.2x digital zoom, 800x800 pixels resolution, 488 nm at 3-8% power, 546 nm at 9-45%power and 647 nm at 9-45% power depending on expression level and fluorochrome, but kept at a constant level throughout the experiments. Images were processed with Adobe Photoshop and Premiere (Adobe systems Inc., CA, USA), ImageJ (NIH, USA) and Imaris (Bitplane AG, Zurich, Switzerland).

Iodination:
The anti I-Ak antibody H116-32 was iodinated using IODO-Gen pre-coated iodination tubes (Pierce, IL, USA) as described by the manufacturer. Briefly 100μg protein was incubated with 40 MBq $Na^{125}I$ for 1 hour at room temperature. Iodinated proteins were separated from free $Na^{125}I$ and unincorporated $^{125}I_2$ on sephadex G-25M columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The specific activity of the labeled proteins was determined by trichloracetic acid precipitation. The amount of soluble radioactivity in the fractions used was less than 5% of total radioactivity.

Quantification of surface bound I-Ak:
For quantification of surface bound I-Ak, $MDCK_{I\text{-}Ak/Ii\text{-}wt}$ cells were seeded at $5x10^5$ cells per well in a 6 well, and incubated with 25μM $CdCl_2$ at different time points. The cells were washed twice in ice cold PBS and incubated on ice with 125I-H116-32 in 0.5 mL PBS (2μg/mL) for 45 minutes. Unbound antibody was removed by washing twice in ice cold PBS with 1%BSA. Cell surface bound antibody was collected by treatment with 0.5M acetic acid in 0.15M NaCl (pH2.5) for 7minutes. To ensure that no antibody was internalized during incubation on ice, the cells were also lysed in 1M NaOH for 10 minutes. Lysate and acid wash were counted in a COBRA γ counter (Packard Instrument Company, Meriden, CT, USA). 10μg/mL cyclohexamide was added to the cells for up to 6 hours to inhibit protein synthesis. For the I-Ak internalization experiments, $MDCK_{I\text{-}Ak/Ii\text{-}wt}$ cells were seeded and treated essentially as above, however after the PBS/BSA wash, the cells were chased in complete medium at 37ºC for different time periods. Then the cells were chilled on ice, washed and lysed as above. Internalized antibody was calculated as the antibody resistant to low pH acid wash relative to the antibody bound before the onset of the chase period.

Antigen presentation:
$MDCK_{I\text{-}Ak/Ii\text{-}wt}$ were used as antigen presenting cells and seeded at $5x10^3$cells/well in 96 well plates, to avoid polarization. The cells were allowed to adhere over night before incubation with 25μM $CdCl_2$ for 2, 6 and 24 hours to induce expression of Ii. The cells were then fixed in 1% PFA for 20 minutes at room temperature, washed extensively and subsequently incubated for 24 hours at 37ºC with 10μg/mL HEL 116-129 and $5x10^4$ cells/well 1B9 T cell hybridomas. T cell stimulatory capacity was measured indirectly by production of IL-2 and proliferation of CTLL-2 cells (IL-2 dependant) which were pulsed with 1μCi 3H-thymidine over night, harvested and counted in a Matrix96 β-counter (Packard Instrument Company, Meriden, CT, USA).

We thank Dmitrii Rodionov for critical reading of the manuscript.

Anderson, M.S., and J. Miller. 1992. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc Natl Acad Sci U S A*. 89:2282-6.

Anderson, M.S., K. Swier, L. Arneson, and J. Miller. 1993. Enhanced antigen presentation in the absence of the invariant chain endosomal localization signal. *J Exp Med*. 178:1959-69.

Bakke, O., and B. Dobberstein. 1990. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell*. 63:707-16.

Bremnes, B., T. Madsen, M. Gedde-Dahl, and O. Bakke. 1994. An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalization. *J Cell Sci*. 107 (Pt 7):2021-32.

Brocke, P., N. Garbi, F. Momburg, and G.J. Hammerling. 2002. HLA-DM, HLA-DO and tapasin: functional similarities and differences. *Curr Opin Immunol*. 14:22-9.

Busch, R., I. Cloutier, R.P. Sekaly, and G.J. Hammerling. 1996. Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *Embo J*. 15:418-28.

Castellino, F., R. Han, and R.N. Germain. 2001. The transmembrane segment of invariant chain mediates binding to MHC class II molecules in a CLIP-independent manner. *Eur J Immunol*. 31:841-50.

Cella, M., A. Engering, V. Pinet, J. Pieters, and A. Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*. 388:782-7.

Cresswell, P. 1994. Assembly, transport, and function of MHC class II molecules. *Annu Rev Immunol*. 12:259-93.

Dugast, M., H. Toussaint, C. Dousset, and P. Benaroch. 2005. AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. *J Biol Chem*. 280:19656-64.

Freisewinkel, I.M., K. Schenck, and N. Koch. 1993. The segment of invariant chain that is critical for association with major histocompatibility complex class II molecules contains the sequence of a peptide eluted from class II polypeptides. *Proc Natl Acad Sci U S A*. 90:9703-6.

Fremont, D.H., D. Monnaie, C.A. Nelson, W.A. Hendrickson, and E.R. Unanue. 1998. Crystal structure of I-Ak in complex with a dominant epitope of lysozyme. *Immunity*. 8:305-17.

Gorvel, J.P., J.M. Escola, E. Stang, and O. Bakke. 1995. Invariant chain induces a delayed transport from early to late endosomes. *J Biol Chem*. 270:2741-6.

Gregers, T.F., T.W. Nordeng, H.C. Birkeland, I. Sandlie, and O. Bakke. 2003. The cytoplasmic tail of invariant chain modulates antigen processing and presentation. *Eur J Immunol*. 33:277-86.

Hansen, H.J., G.L. Ong, H. Diril, A. Valdez, P.A. Roche, G.L. Griffiths, D.M. Goldenberg, and M.J. Mattes. 1996. Internalization and catabolism of radiolabelled antibodies to the MHC class-II invariant chain by B-cell lymphomas. *Biochem J*. 320 (Pt 1):293-300.

Henne, C., F. Schwenk, N. Koch, and P. Moller. 1995. Surface expression of the invariant chain (CD74) is independent of concomitant expression of major histocompatibility complex class II antigens. *Immunology*. 84:177-82.

Humbert, M., P. Bertolino, F. Forquet, C. Rabourdin-Combe, D. Gerlier, J. Davoust, and J. Salamero. 1993. Major histocompatibility complex class II-restricted presentation of secreted and endoplasmic reticulum resident antigens requires the invariant chains and is

sensitive to lysosomotropic agents. *Eur J Immunol*. 23:3167-72.

Kampgen, E., N. Koch, F. Koch, P. Stoger, C. Heufler, G. Schuler, and N. Romani. 1991. Class II major histocompatibility complex molecules of murine dendritic cells: synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture. *Proc Natl Acad Sci U S A*. 88:3014-8.

Kang, S., L. Liang, C.D. Parker, and J.F. Collawn. 1998. Structural requirements for major histocompatibility complex class II invariant chain endocytosis and lysosomal targeting. *J Biol Chem*. 273:20644-52.

Koch, N., G.J. Hammerling, N. Tada, S. Kimura, and U. Hammerling. 1982. Cross-blocking studies with monoclonal antibodies against I-A molecules of haplotypes b, d and k. *Eur J Immunol*. 12:909-14.

Kuchtey, J., M. Pennini, R.K. Pai, and C.V. Harding. 2003. CpG DNA induces a class II transactivator-independent increase in class II MHC by stabilizing class II MHC mRNA in B lymphocytes. *J Immunol*. 171:2320-5.

Kvist, S., K. Wiman, L. Claesson, P.A. Peterson, and B. Dobberstein. 1982. Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell*. 29:61-9.

Lampson, L.A., and R. Levy. 1980. Two populations of Ia-like molecules on a human B cell line. *J Immunol*. 125:293-9.

Landmann, S., A. Muhlethaler-Mottet, L. Bernasconi, T. Suter, J.M. Waldburger, K. Masternak, J.F. Arrighi, C. Hauser, A. Fontana, and W. Reith. 2001. Maturation of dendritic cells is accompanied by rapid transcriptional silencing of class II transactivator (CIITA) expression. *J Exp Med*. 194:379-91.

Machamer, C.E., and P. Cresswell. 1982. Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J Immunol*. 129:2564-9.

McCormick, P.J., J.A. Martina, and J.S. Bonifacino. 2005. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proc Natl Acad Sci U S A*. 102:7910-5.

Miller, J., and R.N. Germain. 1986. Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. *J Exp Med*. 164:1478-89.

Moldenhauer, G., C. Henne, J. Karhausen, and P. Moller. 1999. Surface-expressed invariant chain (CD74) is required for internalization of human leucocyte antigen-DR molecules to early endosomal compartments. *Immunology*. 96:473-84.

Neefjes, J. 1999. CIIV, MIIC and other compartments for MHC class II loading. *Eur J Immunol*. 29:1421-5.

Nguyen, Q.V., and R.E. Humphreys. 1989. Time course of intracellular associations, processing, and cleavages of Ii forms and class II major histocompatibility complex molecules. *J Biol Chem*. 264:1631-7.

Nijenhuis, M., J. Calafat, K.C. Kuijpers, H. Janssen, M. de Haas, T.W. Nordeng, O. Bakke, and J.J. Neefjes. 1994. Targeting major histocompatibility complex class II molecules to the cell surface by invariant chain allows antigen presentation upon recycling. *Eur J Immunol*. 24:873-83.

Nordeng, T.W., T.F. Gregers, T.L. Kongsvik, S. Meresse, J.P. Gorvel, F. Jourdan, A. Motta, and O. Bakke. 2002. The cytoplasmic tail of invariant chain regulates endosome fusion and morphology. *Mol Biol Cell*. 13:1846-56.

Odorizzi, C.G., I.S. Trowbridge, L. Xue, C.R. Hopkins, C.D. Davis, and J.F. Collawn. 1994. Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *J Cell Biol*. 126:317-30.

O'Sullivan, D.M., D. Noonan, and V. Quaranta. 1987. Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. *J Exp Med*. 166:444-60.

Pai, R.K., D. Askew, W.H. Boom, and C.V. Harding. 2002. Regulation of class II MHC expression in APCs: roles of types I, III, and IV class II transactivator. *J Immunol*. 169:1326-33.

Patterson, G.H., and J. Lippincott-Schwartz. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science*. 297:1873-7.

Peijnenburg, A., M.J. Van Eggermond, S.J. Gobin, R. Van den Berg, B.C. Godthelp, J.M. Vossen, and P.J. Van den Elsen. 1999. Discoordinate expression of invariant chain and MHC class II genes in class II transactivator-transfected fibroblasts defective for RFX5. *J Immunol*. 163:794-801.

Pierre, P., and I. Mellman. 1998. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell*. 93:1135-45.

Pieters, J., O. Bakke, and B. Dobberstein. 1993. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J Cell Sci*. 106 (Pt 3):831-46.

Pinet, V., M. Vergelli, R. Martin, O. Bakke, and E.O. Long. 1995. Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature*. 375:603-6.

Pinet, V.M., and E.O. Long. 1998. Peptide loading onto recycling HLA-DR molecules occurs in early endosomes. *Eur J Immunol*. 28:799-804.

Rabinowitz, J.D., M. Vrljic, P.M. Kasson, M.N. Liang, R. Busch, J.J. Boniface, M.M. Davis, and H.M. McConnell. 1998. Formation of a highly peptide-receptive state of class II MHC. *Immunity*. 9:699-709.

Rescigno, M., S. Citterio, C. Thery, M. Rittig, D. Medaglini, G. Pozzi, S. Amigorena, and P. Ricciardi-Castagnoli. 1998. Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc Natl Acad Sci U S A*. 95:5229-34.

Roche, P.A., and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 345:615-8.

Roche, P.A., C.L. Teletski, E. Stang, O. Bakke, and E.O. Long. 1993. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc Natl Acad Sci U S A*. 90:8581-5.

Romagnoli, P., and R.N. Germain. 1994. The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J Exp Med*. 180:1107-13.

Romagnoli, P., C. Layet, J. Yewdell, O. Bakke, and R.N. Germain. 1993. Relationship between invariant chain expression and major histocompatibility complex class II transport into early and late endocytic compartments. *J Exp Med*. 177:583-96.

Rovere, P., V.S. Zimmermann, F. Forquet, D. Demandolx, J. Trucy, P. Ricciardi-Castagnoli, and J. Davoust. 1998. Dendritic cell maturation and antigen presentation in the absence of invariant chain. *Proc Natl Acad Sci U S A*. 95:1067-72.

Salamero, J., M. Humbert, P. Cosson, and J. Davoust. 1990. Mouse B lymphocyte specific endocytosis and recycling of MHC class II molecules. *Embo J*. 9:3489-96.

Sekaly, R.P., C. Tonnelle, M. Strubin, B. Mach, and E.O. Long. 1986. Cell surface expression of class II histocompatibility antigens occurs in the absence of the invariant chain. *J Exp Med*. 164:1490-504.

Shih, N.Y., I. Soesilo, and G. Floyd-Smith. 1997. Stabilization of invariant chain mRNA by 12-O-tetradecanoylphorbol-13-acetate is blocked by IFN-gamma in a murine B lymphoma cell line. *J Interferon Cytokine Res*. 17:747-55.

Simonsen, A., F. Momburg, J. Drexler, G.J. Hammerling, and O. Bakke. 1993. Intracellular distribution of the MHC class II molecules and the associated invariant chain (Ii) in different cell lines. *Int Immunol*. 5:903-17.

Simonsen, A., K.W. Pedersen, T.W. Nordeng, A. von der Lippe, E. Stang, E.O. Long, and O. Bakke. 1999. Polarized

transport of MHC class II molecules in Madin-Darby canine kidney cells is directed by a leucine-based signal in the cytoplasmic tail of the beta-chain. *Journal of Immunology*. 163:2540-8.

Simonsen, A., E. Stang, B. Bremnes, M. Roe, K. Prydz, and O. Bakke. 1997. Sorting of MHC class II molecules and the associated invariant chain (Ii) in polarized MDCK cells. *J Cell Sci*. 110 (Pt 5):597-609.

Stang, E., and O. Bakke. 1997. MHC class II-associated invariant chain-induced enlarged endosomal structures: a morphological study. *Exp Cell Res*. 235:79-92.

Steimle, V., C.A. Siegrist, A. Mottet, B. Lisowska-Grospierre, and B. Mach. 1994. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science*. 265:106-9.

Strubin, M., C. Berte, and B. Mach. 1986. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *Embo J*. 5:3483-8.

Thayer, W.P., L. Ignatowicz, D.A. Weber, and P.E. Jensen. 1999. Class II-associated invariant chain peptide-independent binding of invariant chain to class II MHC molecules. *J Immunol*. 162:1502-9.

Triantafilou, K., M. Triantafilou, K.M. Wilson, R.J. Cherry, and N. Fernandez. 1999. Intracellular and cell surface heterotypic associations of human leukocyte antigen-DR and human invariant chain. *Hum Immunol*. 60:1101-12.

Trombetta, E.S., M. Ebersold, W. Garrett, M. Pypaert, and I. Mellman. 2003. Activation of lysosomal function during dendritic cell maturation. *Science*. 299:1400-3.

Trombetta, E.S., and I. Mellman. 2005. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol*. 23:975-1028.

Vacchino, J.F., and H.M. McConnell. 2001. Peptide binding to active class II MHC protein on the cell surface. *J Immunol*. 166:6680-5.

Wang, K., P.A. Peterson, and L. Karlsson. 1997. Decreased endosomal delivery of major histocompatibility complex class II-invariant chain complexes in dynamin-deficient cells. *J Biol Chem*. 272:17055-60.

Watts, C. 2001. Antigen processing in the endocytic compartment. *Curr Opin Immunol*. 13:26-31.

Wilson, N.S., D. El-Sukkari, and J.A. Villadangos. 2004. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood*. 103:2187-95.

Wilson, N.S., and J.A. Villadangos. 2005. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. *Adv Immunol*. 86:241-305.

Wraight, C.J., P. van Endert, P. Moller, J. Lipp, N.R. Ling, I.C. MacLennan, N. Koch, and G. Moldenhauer. 1990. Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J Biol Chem*. 265:5787-92.

Zhong, G., P. Romagnoli, and R.N. Germain. 1997. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. *J Exp Med*. 185:429-38.